Docket No.: 377882001500
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Stephen TUCK et al.

Application No.: 09/713,136 Confirmation No.: 3530

Filed: November 14, 2000 Art Unit: 1644

For: IMMUNOMODULATORY COMPOSITIONS CONTAINING AN IMMUNOSTIMULATORY SEQUENCE LINKED TO ANTIGEN AND METHODS OF USE THEREOF Examiner: P. Huynh

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is an Appeal from the final rejection mailed July 12, 2005, finally rejecting claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101, and 106-108 under 35 U.S.C. §112, first and second paragraphs. A Notice of Appeal was filed on January 12, 2006. This brief is filed within seven months of filing the Notice of Appeal, and is in furtherance of said Notice of Appeal. In accordance with MPEP §1205.01, the two-month period set by 37 C.F.R. §41.37(a) for filing the Appeal Brief after the Notice of Appeal may be extended by up to five months. Appellants submit herewith a petition and fee for a five-month extension of time under 37 C.F.R. §1.136(a), thereby extending the deadline for filing the Appeal Brief to August 12, 2006 (which fell on a Saturday). Accordingly, this Appeal Brief is timely filed.

The fees required under 37 C.F.R. §41.20(b)(2) are dealt with in the accompanying Fee Transmittal.

This brief contains items under the following headings as required by 37 C.F.R. §41.37 and M.P.E.P. §1206:

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is the assignee of record, Dynavax Technologies Corporation, 2929 Seventh Street, Suite 100, Berkeley, CA 94710.

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no other pending appeals, interferences, or judicial proceedings known to Appellant, Appellant's undersigned attorney, or assignee which will directly affect or be directly affected by, or have a bearing on, a decision by the Board of Patent Appeals and Interferences in the presently pending appeal.

III. STATUS OF CLAIMS

A. Total Number of Claims in Application

There are 66 claims pending in this application.

B. Current Status of Claims

1. Claims canceled: 1-10, 43-62, 64, 87, 90-94, 98, 102-105

2. Claims withdrawn from consideration but not canceled: 11-42, 65-70, 73, 76-82, 85, 88-89

3. Claims pending: 11-42, 63, 65-86, 88-89, 95-97, 99-101, 106-108

4. Claims allowed: none

5. Claims rejected: 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101, 106-108

C. Claims On Appeal

The claims on appeal are claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101, and 106-108.

IV. STATUS OF AMENDMENTS

Applicant filed an Amendment After Final Rejection on January 12, 2006. The Examiner responded to the Amendment After Final Rejection in an Advisory Action mailed February 22, 2006. In the Advisory Action, the Examiner indicated that Applicants' proposed amendments to claims 63, 75, and 108, would not be entered. Accordingly, the claims that appear in Appendix A do not incorporate the amendments to claims 63, 75, and 108, as indicated in the paper filed on January 12, 2006.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention is based on the discovery that the ratio of immunostimulatory sequence (ISS) polynucleotide to antigen in a conjugate molecule can alter the immunostimulatory and biological activities of the conjugate molecule. For example, as the ratio of ISS to antigen increases for a population of conjugate molecules, the allergenicity of the molecules decreases, as does the ability of the molecules to stimulate antibody production.

The specification describes populations of ISS-antigen conjugate molecules with varying immunological activities. For ease of description, ISS-antigen conjugate populations may be divided into three general classes, denoted in the specification as "L" (low extent of conjugation), "M" (medium extent of conjugation), and "H" (high extent of conjugation). "L" conjugates induce strong Th1 responses, induce the highest antibody responses, and provide the least reduction in allergenicity. "M" conjugates induce strong Th1 responses, induce moderate antibody responses, and provide moderate reduction in allergenicity. "H" conjugates induce strong Th1 responses, induce very low antibody responses, and provide the highest reduction in allergenicity. All three forms of the conjugates induce cytotoxic T cell activity. Thus, the different conjugate populations are useful in different types of applications, depending on the immunomodulatory properties desired. L-form conjugates could be anticipated to be most useful in applications where a Th1 response is desired along with high antibody responses, such as in infectious disease vaccines. H-form conjugates could be anticipated to be most useful where strong Th1 responses are desired without high antibody titers, such as in allergy immunotherapy or treatment of certain cancers. M-form conjugates could be anticipated to be most useful in applications where a balance between Th1 cellular immune responses and antibody responses are desired.

Conjugate populations of the "H" type are currently under consideration in this application.

A. Claims under consideration

There are three independent claims under consideration in the application on appeal, claims 63, 75, and 108.

Claim 63

Claim 63 is directed to a population of conjugate molecules that comprise an allergen and a polynucleotide comprising an immunostimulatory sequence (ISS) comprising 5'-cytosine guanine-3', wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length, and wherein the extent of conjugation in the population provides an average of at least 5.5 ISS-containing polynucleotides per allergen molecule.

Dependent claims add limitations that further specify sequence requirements for the ISS or the nature of the allergen, or recite a composition comprising the population recited in claim 63 in a pharmaceutically acceptable excipient.

Claim 75

Claim 75 is directed to a population of conjugate molecules that comprise an allergen and a polynucleotide comprising an immunostimulatory sequence (ISS) comprising 5'-cytosine guanine-3', wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length, and wherein the extent of conjugation in the population provides a ratio of (i) average mass of ISS-containing polynucleotide to (ii) average mass of allergen of at least about 45 to about 40.

Dependent claims add limitations that further specify sequence requirements for the ISS or the nature of the allergen, or recite a composition comprising the population recited in claim 75 in a pharmaceutically acceptable excipient.

Claim 108

Claim 108 is directed to a population of conjugate molecules made by a process comprising combining a polynucleotide comprising an ISS comprising 5'-cytosine guanine-3' and allergen at a ratio of about 17 molar equivalents of the polynucleotide to about 1 molar equivalent of the allergen, wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length, whereby conjugate molecules comprising the polynucleotide and allergen are formed.

There are no pending claims dependent from claim 108.

B. Support and significance of claim terms

The support in the specification and significance of each of the features recited in the independent claims is discussed below:

Population of conjugate molecules A "population of conjugate molecules" is a group of ISS-allergen conjugates (*i.e.*, ISS linked, or attached, to allergen), as described on page 14, lines 14-24. The specification states that "[f]or purposes of this invention, it is understood that such

populations do not necessarily have, and may not have, a constant number of ISS attached to each antigen molecule. Typically, a given population will have a distribution of molecular weights (based on varying extent of conjugation within a given population) and thus an average number of ISS conjugated to antigen" (page 14, lines 15-20).

Allergen The term "allergen" refers to an antigen or antigenic portion of a molecule which elicits an allergic response upon exposure to a subject, as described on page 18, lines 12-18.

Polynucleotide A "polynucleotide" is described on page 15, lines 11-22 as a polymer of nucleosides joined, generally, through phosphodiester linkages. A polynucleotide is described as including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), and modified forms thereof.

Immunostimulatory sequence ("ISS") The terms "immunostimulatory sequence" and "ISS" refer to a polynucleotide sequence that effects a measurable immune response as measured *in vitro*, *in vivo*, and/or *ex vivo*, as described on page 14, lines 4-13. The term "ISS" is also used in the specification to refer to an ISS-containing polynucleotide (page 14, lines 12-13).

Comprising 5'-cytosine guanine-3' The ISS generally comprises the sequence 5'-cytosine,guanine-3', as described on page 36, lines 21-22. A number of embodiments of CG-containing ISS sequences are described on page 36, line 23 – page 38, line 2.

Greater than 8 and less than about 200 nucleotides in length Support for an ISS containing polynucleotide that is greater than 8 and less than about 200 nucleotides in length is provided on page 43, lines 1-7.

Average of at least 5.5 ISS-containing polynucleotides per allergen molecule As described on page 14, line 25 – page 15, line 5, the "average" of a given parameter in a given population refers to "the total of that parameter for the entire population divided by the number of members of the population. For example, the average number of ISS-containing polynucleotides attached to antigen refers to the average number of ISS-containing polynucleotides per antigen

molecule in a population of conjugate molecules (*i.e.*, total number of ISS-containing polynucleotides divided by total number of antigen molecules)." Support for an "average of at least 5.5 ISS-containing polynucleotides per allergen molecule" is provided on page 23, lines 12-13.

Ratio of (i) average mass of ISS-containing polynucleotide to (ii) average mass of allergen of at least about 45 to about 40 Support for a "ratio of (i) average mass of ISS-containing polynucleotide to (ii) average mass of allergen of at least about 45 to about 40" is provided on page 23, lines 14-15.

Ratio of about 17 molar equivalents of the polynucleotide to about 1 molar equivalent of the allergen Support for a "ratio of about 17 molar equivalents of the polynucleotide to about 1 molar equivalent of the allergen is provided on page 30, line 27 – page 31, line 3.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The grounds of rejection on Appeal are:

(1) whether one of skill in the art would be able to practice the invention of claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108 without undue experimentation, in accordance with the enablement requirement of 35 U.S.C. §112, first paragraph.

(2) whether claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101, and 106-108 are described in the specification in such a way as to reasonably convey to one of skill in the art that the inventors had possession of the claimed invention at the time the application was filed, in accordance with the written description requirement of 35 U.S.C. §112, first paragraph.

(3) whether claims 71, 83, 96, and 100 are clear and definite, in accordance with 35 U.S.C. §112, second paragraph.

VII. ARGUMENT

A. The specification provides adequate guidance to enable claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108, in accordance with the enablement requirement of 35 U.S.C. §112, first paragraph.

With respect to this rejection, claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108 stand or fall together.

The Examiner alleges that claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108 are not enabled by the specification. The Examiner concedes that the specification is enabling for a population of conjugate molecules comprising ragweed pollen allergen Amb a1 and an immunostimulatory sequence (ISS) consisting of a sequence selected from the group consisting of SEQ ID NOs:1-8, or the polynucleotide sequences set forth in dependent claims 71, 72, 83, or 84, with an extent of conjugation or ratio of average mass of ISS-containing polynucleotide to allergen as claimed in claims 63 or 75, or a composition comprising such a population in a pharmaceutically acceptable excipient. The Examiner also concedes that the specification is enabling for a population of conjugate molecules made by the process comprising combining a polynucleotide consisting of an ISS of SEQ ID NO:1 and an allergen at a ratio of about 17 molar equivalents of polynucleotide to about 1 molar equivalent of the allergen. 7/12/05 Office Action, pages 3-4.

The Examiner contends that the specification is not enabling for an ISS greater than 8 and less than about 200 nucleotides in length and comprising the sequence 5'-cytosine guanine-3', conjugated to an allergen, as set forth in claims 63, 75, and 108, an ISS comprising the sequence 5'-purine, purine, C,G, pyrimidine, pyrimidine, C,G-3', as set forth in claims 71 and 83, an ISS comprising a sequence as set forth in claims 72 and 84, a mammal allergen as set forth in claims 96 and 100, or a polypeptide antigen as set forth in claims 106 and 107. 7/12/05 Office Action, page 3.

The Examiner alleges that "[t]he specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in **scope** with these claims." 7/12/05 Office Action, page 3, emphasis in original. Applicants disagree.

1. The Examiner has not established a prima facie case for lack of enablement.

"To be enabling, the specification of a patent must teach those skilled in the art to make and use the full scope of the claimed invention without 'undue experimentation' . . . Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples." *See In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). With respect to the enablement requirement for patentability, the burden is on the Examiner to show that the specification is not enabling. MPEP § 2164.04 states that "[a] specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." The MPEP cites the decision in *In re Murdoch*, 439 F.2d 220, 224 (CCPA 1971), in which the court stated that the Patent Office, when making a rejection on the basis of non-enablement, must explain why it doubts the truth or accuracy of the disclosure by backing up its assertion with acceptable contrary evidence or reasoning.

The Examiner has failed to meet the burden of showing that the specification does not provide an enabling disclosure. Applicants respectfully submit that the specification provides all the information required for one of skill in the art to make the claimed ISS-allergen conjugates and to use the conjugates to modulate an immune response.

a. The specification teaches how to make the claimed ISS-containing polynucleotides, conjugates with an allergen thereof, and populations of conjugate molecules as claimed.

The specification teaches the requirements for the ISS and the claimed ISS-containing polynucleotides, and provides methods by which the ISS can be made and evaluated for immunomodulatory activity. See, for example, page 36, line 9, to page 43, line 7. The specification states that "ISS have been described in the art and *may be readily identified using standard assays* which indicate various aspects of the immune response, such as cytokine secretion, antibody

production, NK cell activation and T cell proliferation." Page 36, lines 13-15, emphasis added. The specification provides a number of references that describe ISS, methods for identifying ISS, and methods for evaluating immunomodulatory activity of ISS. (*See* page 4, line 18 – page 6, line13; and page 36, lines 16-20.)

The specification discloses sequence requirements for the ISS of the claimed polynucleotide and provides specific examples of ISS sequences. On page 36, lines 21-26, the specification teaches that the ISS "generally comprises the sequence 5'-cytosine, guanine-3', more particularly comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3'). . . .An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'." The specification at page 37, line 3 – page 38, line 2, goes on to disclose more than 75 specific embodiments of sequences that the ISS may comprise.

The specification teaches that "[t]he ISS *can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art* including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel et al. (1987); and Sambrook et al. (1989)." Page 38, lines 25-28, emphasis added. Synthesis of an ISS would be routine in the art.

The specification also describes allergens and how to make ISS-allergen conjugates. Examples of allergens are described extensively in Table 1, on pages 44-47. Methods for producing ISS-antigen conjugates are described on page 50, line 24 – page 53, line 28. The techniques that are described for conjugation of an ISS-containing polynucleotide to an antigen are standard methods in the art, for example, linking of the polynucleotide to a peptide antigen containing an amino acid with a reactive group, for example, an N-hydroxysuccinimide ester linkage with the N^4 amino group of a cytosine residue (page 50, line 29 – page 51, line 2), linking at the 3' end of the polynucleotide to a peptide via solid support chemistry (page 51, lines 9-26), or attachment at the 5 end of the polynucleotide via an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis (page 51, line 27 – page 52, line 10). Standard methodologies are also described for linkage of the polynucleotide to a lipid (page 53, lines 3-9), or to an oligosaccharide (page 53, lines 10-14). Non-covalent linkage techniques that are routine in the art

are also described, such as linkage via formation of a biotin-streptavidin complex (page 52, lines 14-19) or via ionic interactions between the ISS and residues within the antigen, such as charged amino acids, or through use of a linker portion comprising charged residues that can interact with both the oligonucleotide and the antigen (page 52, lines 20-25).

A method for producing a population of conjugate molecules as claimed is described and exemplified in Example 1, on pages 71-73. Conjugates in accordance with the claimed populations of conjugate molecules were prepared with ragweed allergen Amb a 1 and ISS-containing polynucleotide SEQ ID NO:1. Conjugates of SEQ ID NO:1 and ovalbumin, "prepared essentially as described in Example 1," are also exemplified in Example 6, pages 84-85.

In summary, the specification teaches sequence requirements for ISS and specific examples of ISS, and provides information regarding how to identify and evaluate other ISS using techniques that are well known in the art. Synthesis of ISS and covalent conjugation to an antigen, and production of a population of conjugate molecules as claimed, may also be achieved using techniques that are described and exemplified in the specification and that are standard in the art. Thus, the specification provides adequate guidance regarding how to make ISS, the claimed conjugates comprising an allergen and a polynucleotide comprising an ISS, and a population of conjugate molecules as claimed.

b. The specification teaches how to use the claimed populations of conjugate molecules.

The specification provides guidance regarding administration of the claimed conjugate molecules. For example, suitable formulations and routes for administration are disclosed on pages 54-66. Methods to assess modulation of an immune response after administration of the claimed conjugate molecules are described on pages 66-69. Analysis of the immune response may be assessed, for example, by "measuring antigen-specific antibody production (including measuring specific antibody subclasses), activation of specific populations of lymphocytes such as $CD4^+$ T cells or NK cells, production of cytokines such as IFNγ, IL-2, IL-4, IL-5, IL-10 or IL-12 and/or release of histamine." Page 66, lines 21-24. The specification teaches that standard methods in the art may be used to perform such measurements, such as enzyme-linked immunosorbent assay

(ELISA), fluorescence-activated cell sorting (FACS), or cytotoxicity assays. (See page 66, line 24 – page 67, line 5.)

In addition, the specification provides working examples. Although working examples are not required for enablement (MPEP §2164.02), the claimed invention is exemplified in seven working examples described on pages 71-86 of the specification. Examples 1-5 teach how to prepare populations of conjugate molecules as claimed, containing Amb a 1 allergen and ISS polynucleotide with the sequence depicted in SEQ ID NO:1, and demonstrate different effects with respect to Th1 and Th2 immunological responses using "L," "M," and "H" populations containing different ratios of ISS-containing polynucleotide to antigen, as described in the specification. Example 6 demonstrates ability of ovalbumin-ISS "L," "M," and "H" conjugates to induce cytotoxic T lymphocyte activity. Example 7 demonstrates differing ability of Amb a 1–ISS "L," "M," and "H" conjugates to compete with binding of Amb a 1 specific IgE to Amb a 1 in a competition ELISA assay. All of the uses demonstrated in the working examples exemplify and enable the claimed conjugate populations.

c. It would not require undue experimentation to make and use the claimed invention.

As discussed in detail above, the specification provides guidance regarding *how to make* ISS and populations of ISS-allergen conjugates as claimed. Such techniques are standard in the art. Moreover, sequence requirements for ISS are set forth in the specification, as well as over 75 examples of specific ISS polynucleotide sequences, and methods for identifying and testing additional ISS are described in the specification and are well known and available in the scientific literature. Methods for *how to use* to the claimed populations of conjugates to modulate an immune response are described in detail in the specification, in terms of formulations and routes of administration, as well as testing for modulation of an immune response, using standard techniques in the art. In addition, working examples are provided demonstrating modulation of an immune response using a population of ISS-allergen conjugate molecules as claimed

For a *prima facie* case of non-enablement, the burden is on the Office to demonstrate that there is a reasonable basis to question the presumptively sufficient disclosure made by the

applicant. *See*, for example, *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993). Applicants respectfully submit that the Examiner has not produced adequate evidence to support a lack of enablement, *i.e.*, to establish that with the teachings provided in the specification, a person skilled in the art could not make and use a population of conjugate molecules comprising an ISS-containing polynucleotide greater than 8 and less than about 200 nucleotides in length and comprising a CG sequence conjugated to an allergen with the claimed extent of conjugation, mass of ISS-containing polynucleotide to mass of allergen, or ratio of molar equivalents of ISS-containing polynucleotide to allergen as claimed.

"The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v.Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). Applicants submit that in the instant case, enablement is provided by the disclosure in the specification, and also by knowledge in the art about ISS polynucleotides and allergens.

The Examiner states that "[t]he specification discloses only eight specific immunostimulatory (ISS) consisting of the nucleotide sequence selected from the group consisting of SEQ ID NO: 1-8." 7/12/05 Office Action, page 3. Applicants disagree with this statement by the Examiner. Contrary to the Examiner's assertion that only eight specific immunostimulatory sequences are disclosed in the specification, pages 36-43 of the specification provide over 75 examples of ISS for use in the invention, as well as methods for making additional ISS-containing polynucleotides. At pages 66-69, the specification provides methods by which the skilled artisan can assess the activity of any ISS-containing polynucleotide. Applicants also submit that ISS comprising a CG dinucleotide were well known the in the art at the time the application was filed. A review of the many references regarding CG-containing immunostimulatory sequences cited in the specification and submitted to the Office clearly shows that a CG dinucleotide is a necessary element of the claimed category of immunostimulatory sequences. Such extensive disclosure provides adequate guidance such that a skilled artisan would be able to practice the invention without undue experimentation.

The Examiner further asserts that "[t]he specification does not teach how to make any population of conjugate molecules . . . because the term 'comprising' or 'comprises' is open-ended. It expands the immunostimulatory sequence (ISS) to include additional undisclosed nucleotides at either or both ends so long the nucleotide sequence has a 5' cytosine and a 3' guanine. In addition to the problem of the undisclosed ISS, there is insufficient guidance as to the structure of the polynucleotide that is 'greater than 8 and less than about 200 nucleotides in length' without the nucleotide sequence. Even if the ISS is limited to SEQ ID NO: 1, the specification discloses ISS consisting of SEQ ID NO: 1 is only 22 nucleotides in length. The rest of polynucleotide containing the ISS is not adequate taught in the specification without the nucleotide sequence." 7/12/05 Office Action, page 4.

Applicants disagree with the Examiner's interpretation of the phrase "5'-cytosine guanine-3'" in the claims. The phrase, in conjunction with the term "comprises," which the Examiner concedes is "open-ended" claim language, indicates that the claimed ISS contains a CG sequence within the *within* the ISS polynucleotide sequence, *not* "a 5' cytosine and a 3' guanine" as asserted by the Examiner. This point is discussed in further detail below in the section regarding the indefiniteness rejection under 35 U.S.C. §112, second paragraph. Further, Applicants submit that the inclusion of the term "comprises" with regard to the polynucleotide in the claims leaves the claims fully enabled by the specification. Active immunostimulatory sequences are well known in the art with a variety of flanking sequences and a variety of lengths. Immunostimulatory polynucleotides are well known in the art and polynucleotides with immunostimulatory sequences active in cells of many mammalian species have been described in the scientific literature, including humans, monkeys, chimpanzees, cows, swine, dogs, cats, rabbits, mice, and rats. In particular, much has been described about ISS activity in human cells and immunostimulatory sequences active in human cells have been the subject of much scientific and patent literature. Thus, it would not require undue experimentation to apply the foundation provided by the ISS art to identify additional ISS sequences that will be useful in the practice of the claimed invention.

The Examiner also alleges that the claims are not enabled due to the breadth of the term "allergen" in the independent claims and the terms "pollen allergen," "insect allergen," "mammal allergen," "nut allergen," "crustacean allergen," and "fungal allergen" in dependent claims. The

Examiner states that "[t]he specification discloses only one ragweed allergen Amb a1 conjugated to a polynucleotide consisting of SEQ ID NO: 1." 7/12/05 Office Action, page 3. The Examiner states that "there is insufficient guidance as to the structure of any 'mammal allergen' without the amino acid sequence." 7/12/05 Office Action, page 4. The Examiner further states that "the specification does not teach any and all allergen such as 'mammal allergen', the specification discloses only the specific Fel dI from cat, Bos d2 from Cow, Cn fI and Can f2 from dog, Equ c1 from horse, and mouse urinary protein from mouse (see Table 1, page 45), the specification does not teach all mammal allergen such as allergen from whale, and any allergen from all insects, all pollen, all nut, all crustacean and all fungal allergen." 7/12/05 Office Action, page 8.

The Examiner is incorrect that the specification discloses only one allergen conjugate, containing the ragweed allergen Amb a1. While working examples are not required for enablement, the specification provides working examples with at least two species of antigen-ISS conjugates: conjugates comprising the allergen Amb a1, which the Examiner acknowledges is enabled, and conjugates comprising ovalbumin (OVA). As disclosed at page 85 in Example 6, OVA-ISS conjugates of all classes, *i.e.*, H, M, and L, induced greater CTL activity in mice than antigen alone or than antigen conjugated with non-ISS polynucleotides.

Given the teaching of the specification and the knowledge of one of skill in the art, the Examiner has not provided a reasonable basis for stating that conjugate molecules comprising allergens other than Amb a1 are not enabled. Allergens are well known in the art, with many illustrative allergens disclosed in the specification in Table 1. Contrary to the Examiner's assertions, Applicants submit that it is not necessary for the application to disclose "any and all allergen such as 'mammal allergen'" including "allergen from whale," or all possible allergens from insects, pollen, nuts, crustaceans, and fungi in order to enable the claims. A representative number of allergens are disclosed in the specification (see Table 1), which provides sufficient guidance to enable a skilled artisan to practice the claimed invention. As stated in the specification, preparation of these allergens is generally known in the art, and methods for conjugating an allergen to an ISS-containing polynucleotide are known in the art and described in the specification.

The court in *In re Wands* found that the enablement requirement was satisfied by a "disclosure [that] provides considerable direction and guidance on how to practice [the] invention and presents working examples," in view of the fact that "[t]here was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known." *In re Wands*, 858 F.2d 731, 740 (Fed. Cir. 1988). The court in *United States v. Telectronics* held that "[s]ince one embodiment [was] . . . disclosed in the specification, along with the general manner in which its current range was ascertained, . . . other permutations of the invention could be practiced by those skilled in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 786 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). The Federal Circuit has stated that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988). Applicants respectfully submit that the specification provides a reasonable amount of guidance to the skilled artisan with respect to the direction in which the experimentation should proceed to optimize the teachings of the specification and the art and that any additional necessary experimentation is well within the level of ordinary skill in the art, *i.e.*, no *undue* experimentation is required. Applicants respectfully submit that varying the nucleic acid sequence of oligonucleotides and testing the oligonucleotides for immunostimulatory activity are well within the bounds of routine experimentation by one of skill in the art, as well as conjugation of allergens to such immunostimulatory oligonucleotides to produce a population of conjugate molecules as claimed.

In response to the Examiner's statement on page 2 of the 7/12/05 Office Action that the specification is enabling only for conjugate molecules consisting of an ISS selected from the group consisting of SEQ ID NOs: 1-8, conjugate molecules consisting of the sequence 5' purine, purine, C, G pyrimidine, pyrimidine, C, G 3', and conjugate molecules consisting of the sequences set forth in claims 72 and 84, Applicants disagree that the specification is enabling for only these illustrative examples of ISS recited by the Examiner. For example, a claimed H class conjugate molecule comprising an allergen (such as Amb a1, or any allergen listed in Table 1 at pages 44-47) and an ISS comprising, for example, 5'-cytosine guanine-3' dinucleotide with additional nucleotides at either or both ends, or an ISS comprising 5'-purine, purine, C, G pyrimidine, pyrimidine, C,G-3' with additional nucleotides at either or both ends, or the sequence disclosed in SEQ ID NO:1 with

additional nucleotides at either or both ends can be prepared and tested for function using information provided in the specification and/or known in the art. Methods for preparing such conjugate molecules and testing them are disclosed in the specification and known in the art and do not require undue experimentation.

In response to the Examiner's contention on page 3 of the 7/12/05 Office Action that the specification is enabled only for "a population of conjugate molecules made by the process: combining a polynucleotide consisting of an immunostimulatory sequence (ISS) of SEQ ID NO: 1 and an allergen at a ratio of about 17 molar equivalents of the polynucleotide to about 1 molar equivalent of the allergen whereby conjugate molecules comprising the polynucleotide and allergen are formed, wherein the polynucleotide is consisting of the sequence 5'-cytosine, guanine-3'[1] for treating allergy," Applicants disagree that the specification is enabling for *only* a population of conjugate molecules made by this process. Examples of many ways to couple the ISS-containing polynucleotide and antigen to generate the claimed conjugate populations are provided, for example, at pages 30-32 and 50-53 of the specification. Further, the Examiner alleges that the conjugate molecules made by the process recited are enabled only for treating allergy. Applicants note that the claims under consideration are directed to *compositions, not methods for treating an allergy.*

In response to the Examiner's allegation on page 5 of the 7/12/05 Office Action that the mass of an ISS-containing polynucleotide and mass of an antigen cannot be determined without nucleotide and amino acid sequences, Applicants submit that it would not require undue experimentation to determine the mass of any particular ISS-containing polynucleotide or any particular antigen known in the art, since the masses of nucleotides and amino acids are well known.

In conclusion, given the guidance in the specification and in view of the working examples demonstrating production of the claimed conjugate populations and use for modulating an immune response, it would not require undue experimentation for a skilled artisan to practice the

[1] In this statement by the Examiner, it is unclear to which polynucleotide sequence he is referring. In the first part of the statement, the Examiner refers to "a polynucleotide *consisting of an immunostimulatory sequence (ISS) of SEQ ID NO: 1*" but later in the same statement, the Examiner states that "the polynucleotide is *consisting of the sequence 5'-cytosine, guanine-3'*," which is inconsistent with the sequence consisting of SEQ ID NO:1.

claimed invention. Applicants respectfully submit that the pending claims are in compliance with the enablement requirement and that the Examiner has not established a *prima facie* case for lack of enablement.

2. The references relied upon by the Examiner do not support a lack of enablement.

The Examiner relies on six references in the enablement rejection, none of which supports a finding of non-enablement of the claimed invention.

The Examiner states that "Stryer *et al.*, [*Biochemistry*, 3rd edition, W.H. Freeman and Co., New York, 1988, pages 31-33] . . .teach that a protein is highly dependent on the overall structure of the protein itself and that the primary amino acid sequence determines the conformational of the protein." 7/12/05 Office Action, page 7. The Examiner states that "Ngo *et al.* [*The Protein Folding Problem and Tertiary Structure Prediction*, K. Merz, Jr. and S. Le Grand, Editors, Birkhauser, Boston, 1994, pages 491-495] . . .teach that the amino acid positions within the polypeptide/protein that can tolerate change such as conservative substitution or no substitution, addition or deletion which are critical to maintain the protein's structure/function will require guidance." 7/12/05 Office Action, page 8. The Examiner states that "Chatel et al [(2003) *Allergy* 58:641-647] . . .teach various factors such as allergen structure, mouse strain, CpG/recombinant protein expression influence the immune response." 7/12/05 Office Action, page 8.

Applicants note that the Stryer reference is a general biochemistry text and the pages cited by the Examiner discuss the basic levels of protein structure. The Ngo reference pages cited by the Examiner discuss algorithms for predicting structure of a given protein from its amino acid sequence. The Chatel reference discusses genetic immunization using plasmid DNA. *None of these references provides evidence to support the alleged non-enablement of the invention. None of these references provides evidence that the specification as filed fails to teach how to make and use populations of ISS-allergen conjugate molecules as claimed.* As described in the specification and as discussed in detail above, allergens are well known in the art. Applicants respectfully submit that the specification as filed fully enables the use of allergens in the claimed compositions.

Applicants further submit that neither Stryer et al. nor Ngo et al. should apply to the present enablement rejection because they were previously withdrawn by the Examiner as

supporting this rejection. In the Office Actions mailed December 18, 2002 and July 1, 2003, the Examiner relied upon Stryer et al. and Ngo et al. in the 35 U.S.C. §112, first paragraph, enablement rejection. After Applicants' response filed on October 31, 2003, the Examiner withdrew reliance on Stryer et al. and Ngo et al., since these references were not cited by the Examiner in the Office Action mailed April 21, 2004. In the Office Action mailed December 16, 2004, the Examiner re-cited Stryer et al. and Ngo et al. as supporting the enablement rejection. Since these references were earlier withdrawn, they should not apply as supporting the present enablement rejection.

The Examiner states that "Van Uden *et al* [(1999) *Allergy and Clinical Immunology* 104(5): 902-907] teach even after intensive attempts to precisely define the DNA sequence structure required for immune stimulation, this most fundamental aspect of ISS is only partially understood." 7/12/05 Office Action, page 5. The Examiner states that "Segal *et al* [(2000) *Journal of Immunology* 164(11): 5683-5688] teach that immunostimulatory sequences such as CpG oligonucleotides are potent adjuvant that triggering *autoimmune disease* in predisposed susceptible individual." 7/12/05 Office Action, page 5, emphasis in original. The Examiner states that "Yamada *et al* [(2002) *Journal of Immunology* 169(10):5590-5594] teach that the sequence and length of a DNA strand determine its activity and depending on how the polynucleotide's secondary/tertiary structure are fold, activity may be gained or lost. . . .Yamada et al teach oligonucleotides containing runs of greater than 15 polyGs can inhibit both CpG and mitogen induced immune response. . . .Yamada *et al* also teach the relative locations of the immunostimulatory motif such as CG on a DNA strand determine the magnitude and nature of the resultant immune response and suppression is generally dominant over stimulation. However, when a CpG motif is immediately 5' to a suppressive motif, stimulation dominates. Further, when the distance between motifs exceeds 10 bases, this effect dissipates." 7/12/05 Office Action, page 5.

Applicants note that Van Uden et al. disclose and present numerous CG-containing polynucleotide sequences as "potent immunostimulatory DNA sequences" in Table 1 and go on to state that the dinucleotide sequence 5'-CG-3' is generally required for immunostimulatory activity. See, for example, page 904. Taken in its entirety, Van Uden et al. teach that a CG dinucleotide is a critical element for immunostimulatory activity of the oligonucleotide., *which supports enablement of the invention as claimed*. Segal et al. refer to "CpG-containing oligonucleotides" as

immunostimulatory and the only requirement taught by Segal et al. for the immunostimulatory activity of an olignonucleotide is the presence of a CG dinucleotide. Thus, Segal et al. teach immunostimulatory activity of oligonucleotides containing CG dinucleotides, *which supports enablement of the invention as claimed*. Yamada et al. describe features of DNA sequences which *suppress* immune activation by immunostimulatory DNA, not features of the immunostimulatory DNA itself. Yamada provides nothing to support the alleged lack of enablement of the instant invention. *None of these references provides evidence to support the alleged non-enablement of the invention. None of these references provides evidence that the specification as filed fails to teach how to make and use populations of ISS-allergen conjugate molecules as claimed.*

In view of the cited six references, two of which were previously withdrawn by the Examiner as supporting the enablement rejection, the Examiner states that "[g]iven the unlimited number of polynucleotide 'comprising' an ISS conjugated to unlimited number of allergen such as mammal allergen, there is insufficient working examples demonstrating that the undisclosed population of conjugate molecules are immunostimulatory, let alone in vivo working example that population of conjugated molecules are useful for treating allergy." 7/12/05 Office Action, page 5. In response, Applicants note that no working examples, much less *in vivo* working examples, are necessary to comply with 35 U.S.C. §112, first paragraph. Further, the specification *does* include *in vivo* working examples related to conjugate molecules that demonstrate their immunostimulatory properties (see pages 71-86) and provides disclosure regarding additional examples of conjugate molecules and methods for making, using, and characterizing them. In making these statements, the Examiner has inappropriately dismissed the teachings of the specification, and has not provided acceptable documentation or sound scientific reasoning to support any doubt of the teachings of the specification, as required for a rejection on the basis of lack of enablement. *See, e.g., In re Marzocchi*, 439 F.2d 220, 224 (CCPA 1971). Also, Applicants note that the presently claimed invention under consideration is directed to compositions, not methods of treating allergies, rendering the Examiner's comments in this regard moot.

3. An analysis of the factors set forth in In re Wands shows that the claimed invention is enabled.

MPEP §2164.01(a) lists the factors to be considered in an enablement analysis, as set forth in *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). These factors include (A) the breadth of the claims; (B) the nature of the invention; (C) the state of the prior art; (D) the level of one of ordinary skill; (D) the level of predictability in the art; (F) the amount of direction provided by the inventor; (G) the existence of working examples; and (H) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In the 7/12/05 Office Action at page 6, the Examiner references the *In re Wands* decision, and states "the decision of the court indicates that the more unpredictable the area is, the more specific enablement is necessary. In view of the quantity of experimentation necessary, the limited working examples, the unpredictability of the art, the lack of sufficient guidance in the specification and the breadth of the claims, it would take an undue amount of experimentation for one skilled in the art to practice the claimed invention." However, the Examiner fails to provide an analysis to provide support for these statements.

Applicants provide the following enablement analysis using the factors set forth in *In re Wands*, showing that practice of the claimed invention would not require undue experimentation:

A. With respect to the breadth of the claims, the claims are directed to populations of conjugate molecules comprising an allergen and an ISS-containing polynucleotide, wherein the ISS comprises 5'-cytosine guanine-3' and wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length. Numerous ISS that fall within the parameters set forth in the claims are known in the art and disclosed in the specification, and other ISS within the scope of the claims may be identified and tested using techniques that are well-established in the art. Numerous allergens are also known in the art and disclosed in the specification.

B. With regard to the nature of the invention, the invention relates to populations of ISS-allergen conjugate molecules that are useful for modulating an immune response to an allergen, competing with antigen-specific antibody for binding to antigen, and suppressing histamine release, based on the average extent of conjugation of ISS-containing polynucleotides to allergen.

C and D. The state of the prior art (factor C) and the level of one of ordinary skill (factor D) are high, because much has been written in both the scientific and patent literature about how to make and use immunostimulatory sequences in several species.

E. With regard to the level of predictability in the art, it is predictable that many polynucleotide sequences within the parameters set forth in the claims are operable in the practice of methods described in the specification, as demonstrated by the working examples and the many active immunostimulatory sequences described in the art (see, *e.g.*, Van Uden et al., *supra*, page 903; and references cited in the specification at pages 4-6).

F and G. With respect to the amount of direction provided by the inventor (factor F) and the existence of working examples (factor G), Applicants disclosed working examples in the specification showing how to make populations of conjugate molecules as claimed and use of such populations to modulate an immunological response to an allergen, modulate histamine release, induce cytotoxic T lymphocyte response, and compete with antigen-specific antibody for binding to antigen. Further, guidance is provided in the specification regarding how to make the claimed conjugate molecules and populations of conjugate molecules, how to identify and assess ISS polynucleotides for use in the claimed conjugate molecules, and how to use the claimed populations of conjugate molecules to modulate an immune response to an allergen.

H. With respect to the quantity of experimentation needed to make or use the invention based on the content of the disclosure, a number of ISS polynucleotide sequences are provided in the specification, including one which is exemplified in two different conjugate molecules in working examples, as well as disclosure teaching how to identify and evaluate other immunostimulatory polynucleotide sequences. Further, numerous ISS are known in the art, as well as techniques to test them for immunostimulatory activity, for which references are provided in the specification and incorporated by reference (page 6, lines 21-21). A number of exemplary allergens are described in the specification in Table 1, and two allergens (Amb a 1 and ovalbumin) are exemplified in populations of conjugate molecules as claimed in working examples. Thus, the quantity of experimentation needed to practice the claimed invention is low, based on the disclosure

of how to make and use the claimed conjugate populations both by virtue of description in the specification and exemplification in working examples.

In conclusion and in view of the foregoing, the amount of experimentation needed to make and use the claimed compositions is not undue.

4. Other issued U.S. patents contain claims that recite an ISS comprising 5'-cytosine guanine-3'.

In response to the Examiner's contention that the claims are not enabled due to the scope of the claimed ISS, Applicants noted in their response to the 7/1/03 Office Action that "the Office has recently issued claims directed to methods of treating a subject through administering an immunostimulatory or immunomodulatory polynucleotide comprising an ISS, wherein the ISS comprises the sequence 5'-C,G-3'." 10/31/03 Response, page 16. Claims from the issued patents referred to by Applicants are reproduced below:

Claim 1 of U.S. Patent No. 6,498,148 recites:

> 1. A method for treating asthma, comprising: administering to a mammal sensitized to an asthma-stimulating antigen an immunostimulatory polynucleotide comprising an immunostimulatory sequence (ISS), wherein *the ISS comprises the sequence* ***5'-cytosine-guanine-3'***, wherein the immunostimulatory polynucleotide does not comprises a nucleotide sequence encoding the antigen, and wherein the immunostimulatory polynucleotide is administered without the antigen, including without a polynucleotide encoding the antigen, and in an amount sufficient to treat asthma.

Claims 1 and 2 of U.S. Patent No. 6,534,062 recite:

> 1. A method of increasing antigen-specific T lymphocyte activity in a CD4+ T cell-deficient individual, comprising administering a formulation *comprising an immunostimulatory nucleic acid molecule* and an antigen in an amount effective to increase antigen-specific CTL activity, wherein the immunostimulatory nucleic acid is covalently linked to the antigen.
>
> 2. The method of claim 1, wherein *the immunostimulatory nucleic acid comprises the sequence* ***5' C-G 3'***.

Claim 1 of U.S. Patent No. 6,552,006 recites:

> 1. A method for treating mycobacterial infection in a subject, the method comprising: administering to a subject an immunomodulatory nucleic acid molecule in an amount effective to inhibit intracellular replication of the mycobacterium, wherein *the immunomodulatory nucleic acid comprises an immunostimulatory sequence comprising* ***5' CpG 3'***; and administering to the subject an anti-pathogenic agent in an amount effective to decrease or inhibit growth of the mycobacterium, thereby treating the infection.

Claim 1 of U.S. Patent No. 6,613,751 recites:

> 1. A method for ameliorating gastrointestinal inflammation in a subject comprising: administering to a subject suffering from gastrointestinal inflammation a formulation comprising an immunomodulatory nucleic acid to the subject, *the immunomodulatory nucleic acid comprising the sequence* ***5'-CpG-3'***, wherein said immunomodulatory nucleic acid is isolated or synthetic, said administering being in an amount effective to ameliorate a symptom of gastrointestinal inflammation in the subject; wherein said administering is by a route selected from oral and subcutaneous, and wherein gastrointestinal inflammation is ameliorated in the subject.

The claims above recite an ISS comprising a 5'-C,G-3' sequence, except claim 1 of U.S. Patent No. 6,534,062, which is of even broader scope and recites "an immunostimulatory nucleic acid molecule," with no recited sequence requirements. The claims in these patents are supported with experiments in which a limited number of 5'-C,G-3'-containing oligonucleotides were tested for a particular activity or effect in a mouse model, and in some cases, on human cells in culture. Thus, in these cases, the Office has deemed the state of the art such that the task of identifying nucleotides surrounding the core 5'-C,G-3' motif of an immunostimulatory polynucleotide (or identifying a polynucleotide with immunostimulatory activity, in the case of U.S. Patent No. 6,534,062) would not present an undue burden to the skilled artisan. Each of these patents contains claims reciting an ISS of identical scope and breadth as (or broader than) the ISS of the instant

claims comprising the sequence 5'-C,G-3' and was found by the USPTO to be in compliance with 35 U.S.C. §112, first paragraph.

B. Claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101, and 106-108 are described in the specification in such a way as to reasonably convey to one of skill in the art that the inventors had possession of the claimed invention at the time the application was filed, in accordance with the written description requirement of 35 U.S.C. §112, first paragraph.

With respect to this rejection, claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108 stand or fall together.

The Examiner alleges that claims 63, 71-72, 74-75, 83-84, 86, 95-97, 99-101 and 106-108 are not adequately supported by the written description of the specification. The Examiner states that the specification does not provide a written description of all polynucleotides comprising ISS sequences within the parameters set forth in the pending claims or all allergens, including mammal allergens or polypeptide antigens, as set forth in the claims. 7/12/05 Office Action, page 9. Applicants disagree with the Examiner's contention that the claimed invention fails to satisfy the written description requirement for patentability, as discussed in detail below.

The Examiner states "[w]ith the exception of the specific population of conjugates comprising the specific immunostimulatory sequence (ISS) and the specific allergen disclosed in Table 1, there is inadequate written description about the structure associated with function of all polynucleotide comprising any immunostimulatory sequence (ISS) wherein the polynucleotide is greater than 8 and less than about 200 nucleotide in the claimed conjugate molecules without the nucleotide sequence." 7/12/05 Office Action, page 9. Thus, the Examiner indicates that populations of conjugate molecules comprising specific ISS and the allergens disclosed in Table 1 do comply with the written description requirements of 35 U.S.C. §112, first paragraph.

1. The Examiner has failed to meet the burden required to sustain a written description rejection.

"There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed." MPEP §2163.I.A., citing *In re Wertheim*, 541 F.2d 257, 263 (CCPA 1976). "Consequently, *rejection of an original claim for lack of written description should be rare*." MPEP §2163.03, emphasis added. "The Examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims." MPEP §2163.II.A. The Examiner has failed to meet this burden. The Examiner has not provided supported reasoning as to why the relevant identifying characteristics of the claimed conjugate populations as described in the specification and the description of allergens and ISS-containing polynucleotides provided in the specification are insufficient to satisfy the written description requirement.

For example, the Examiner states that the specification lacks written description support for allergens other than the specific allergens disclosed in Table 1 in the claimed conjugate molecules (7/12/05 Office Action, page 9, lines 18-20; page 11, line 31 – page 12, line 2). The Examiner also states that the term "mammal allergen" lacks written description basis without amino acid sequences. 7/12/05 Office Action, page 9, line 31 – page 10, line 1; page 11, line 29-31. Applicants disagree that the terms "allergen" and "mammal allergen" are not supported by the description. The specification lists many examples of well-known allergens, including mammal allergens, for use in the claimed invention. Although not explicitly presented in the specification, specific information for the allergens, including structural information, was well known in the art at the time the application was filed. See, for example, the allergens and citations listed in Table 1, pages 44-47, including seven mammal allergens listed on page 45. Reference citations are provided for each allergen in the table, all published before the priority date of the present application, and are incorporated by reference in the application (page 6, lines 20-21). Thus, much information about allergens, including amino acid sequences, was known in the art at the time of filing. Applicants submit that the specification in combination with knowledge in the art adequately describes possession of both the claimed genus "allergen" and the claimed subgenus "mammal

allergen." The Examiner has provided no evidence or reasoning to support the assertion that a person skilled in the art would not recognize that the written description of the specification provides support for the terms "allergen" and "mammal allergen" in the claims.

The Examiner has also failed to provide evidence that a person of skill in the art would not recognize that Applicants had possession of the invention with respect to the ISS-containing polynucleotides of the claimed conjugate molecules, given the structural characteristics described in the specification and well known in the art. The present invention involves the use of an ISS which relies on the presence of a 5'-CG-3' dinucleotide for activity. Many sequences which include the 5'-CG-3' sequence as well as additional nucleotide bases are known to provide immunostimulatory activity to the ISS-containing polynucleotide, as described in the specification and known to those of skill in the art. *See, e.g.,* references provided on pages 4-6 of the specification, which are incorporated by reference in the application (page 6, lines 20-21), and ISS sequences disclosed on pages 36-38 of the specification. The Examiner has not provided evidence or reasoning to support the assertion that a person skilled in the art would not recognize that the written description of the specification provides support for the claimed ISS.

"A description as filed is presumed to be adequate, unless or until *sufficient evidence or reasoning to the contrary* has been provided by the examiner to rebut the presumption. The examiner, therefore must have a *reasonable basis* to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a *preponderance of evidence* why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims." MPEP §2163.04, citations omitted, emphasis added. In the instant case, the Examiner has presented no evidence to rebut the presumption that Applicants' disclosure contains adequate written description such that a person skilled in the art would understand that Applicants had possession of the invention as claimed. Thus, the Examiner has failed to meet the burden required for a written description rejection.

2. The claimed invention satisfies the legal standard for written description.

"The purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not; the applicant for a patent is therefore required to

'recount his invention in such detail that his future claims can be determined to be encompassed within his original creation.'" *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1330 (Fed. Cir. 2003), citing *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1561, (Fed. Cir. 1991). "Satisfaction of this requirement is measured by the understanding of the ordinary skilled artisan." *Id.*, citing *Lockwood v. Am. Airlines, Inc.*, 107 F.3d 1565, 1572, (Fed. Cir. 1997). "Compliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Id.*, citing *Enzo Biochem v. Gen-Probe, Inc.*, 196 F.3d 1316, 1324 (Fed. Cir. 2002).

In the instant case, the specification provides a description of sufficient, relevant identifying characteristics of the claimed populations of conjugate molecules such that an ordinary skilled artisan would recognize that the inventor had possession of the claimed invention when the application was filed. With respect to the nature of the invention claimed, immunostimulatory sequences and allergens have been well characterized and are well known in the art. The information provided in the specification with respect to the parameters of the claimed conjugate populations sufficiently describes the structural and functional characteristics of the invention as claimed.

The written description requirement "may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332 (Fed. Cir. 2003). "An adequate written description of the invention may be shown by any description of sufficient, relevant identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." MPEP §2163.II.A.3.(a).

The structure-function relationship of the claimed populations of conjugate molecules is described in the specification. The invention is based on the discovery that the ratio of ISS to antigen, which includes allergen, in a conjugate molecule (*i.e.*, structure) can alter the immunostimulatory and biological activities of the conjugate molecule (*i.e.*, function). For example, as the ratio of ISS to antigen increases for a population of conjugate molecules, the allergenicity of the molecules decreases. Other examples of the structure-function relationship of

conjugate molecules are described in the specification. For example, the H class of conjugate molecules is described in the specification, for example, in the paragraph bridging pages 12-13 ("Conjugates containing high ISS:antigen ratios ("H") induce strong Th1 responses, induce very low antibody responses, and provide the highest reduction in allergenicity." Page 13, lines 3-5.) This structure-function relationship is also exemplified in the working examples of the application.

The invention relies on the unique structure and resultant activity of the claimed conjugate molecules comprising the allergen and ISS and having the recited extent of conjugation (in the case of claim 63), the recited ratio of average mass (in the case of claim 75), or the recited ratio of molar equivalents (in the case of claim 108). Methods of preparing such conjugate populations are described in the specification (pages 50-53 and 71-73). Methods of assessing such conjugate populations are also described (pages 69-71 and 73-86). ISS are well developed in the art. (See, for example, pages 4-6 of the specification, on which are provided numerous references describing ISS.) Further, the art recognizes the claimed 5'-CG-3' motif as essential for ISS activity. (See, for example, Van Uden et al. and Segal et al., *supra*, cited by the Examiner in the enablement rejection, discussed in detail above.) Allergens are also well developed in the art. (See, for example, pages 43-50 of the specification, including Table 1.)

3. It is not necessary to describe every ISS or every allergen, as alleged by the Examiner, to comply with the written description requirement.

The Examiner asserts that the specification does not provide a written description of any ISS comprising the sequence 5'-CG-3' or of any allergen and states that "one of skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus." 7/12/05 Office Action, page 10. The Examiner asserts that written description requires "the sequence itself."

Applicants point out that it is a well-established principle of patent law that "patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art." *In re Vaeck*, 947 F.2d 488, 496 (Fed. Cir. 1991). In *In re Angstadt*, the court considered the issue of whether section 112 requires disclosure of a test with every species covered by a claim and concluded that requirement of such a complete disclosure would necessitate a patent

application with thousands of examples and "would force an inventor seeking patent protection to carry out a prohibitive number of actual experiments." *In re Angstadt*, 537 F.2d 498, 502 (CCPA 1976). The court concluded that such a requirement would be against public policy because it would have the effect of "depriving inventors of claims which adequately protect them and [would limit] them to claims which practically invite appropriation of the invention while avoiding infringement[, which would] inevitably [have] the effect of suppressing disclosure." *Id.* at 504.

In the instant case, Applicants have disclosed representative ISS and allergen species. Numerous other ISS and allergens are known in the art. The USPTO guidelines with respect to written description confirm that it is not necessary for Applicants to disclose every species within the scope of a claim. According to Guidelines presented by Deborah Reynolds in the USPTO TC 1600 West Coast Road Show 2005 regarding written description:

> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, *even if every nuance of the claim is not explicitly described in the specification*, then the requirement for an adequate written description is met. (Emphasis added.)

With respect to the presently claimed invention, given the knowledge in the art with respect to ISS and allergen sequences, it is not necessary for Applicants to have explicitly described each of these sequences to satisfy the written description requirement. A skilled artisan would have understood Applicants to have been in possession of the claimed populations of conjugate molecules in view of the description, the knowledge in the art, and the working examples of the application.

"Possession may be shown in a variety of ways including description of an actual reduction to practice . . . or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention." MPEP §2163.02. Applicants described both actual reduction and distinguishing identifying characteristics of the claimed conjugate populations. Actual reduction to practice is described for two different species of conjugate molecules in the working examples of the application. Further, identifying characteristics are described with respect to the claimed populations of conjugate molecules (average ISS-containing polynucleotide per allergen molecule, ratio of average mass of ISS-containing

polynucleotide to average mass of allergen, or ratio of molar equivalents of ISS-containing polynucleotide to allergen). Identifying characteristics are also described with respect to the claimed ISS. A sequence motif required for immunostimulatory activity, 5'-CG-3', is described in the application and well known in the art (disclosed in numerous references including the Van Uden et al. and Segal et al. references cited by the Examiner in the enablement rejection). Thus, Applicants have adequately described the invention in accordance with 35 U.S.C. §112, first paragraph.

C. Claims 71, 83, 96, and 100 particularly point out and distinctly claim the subject matter which Applicants regard as the invention, in accordance with 35 U.S.C. §112, second paragraph.

With respect to this rejection, claims 71 and 83 stand or fall together, and claims 96 and 100 stand or fall together.

Standard for definiteness of claim language

35 U.S.C. §112, second paragraph, states that the claims of a patent must particularly point out and distinctly claim the subject matter that the applicant regards as the invention. "The primary purpose of this requirement of definiteness of claim language is to ensure that the scope of the claims is clear so the public is informed of the boundaries of what constitutes infringement of the patent." MPEP §2173.

"The examiner's focus during examination of claims for compliance with the requirement for definiteness of 35 U.S.C. 112, second paragraph, is whether the claim meets the threshold requirements of clarity and precision, not whether more suitable language or modes of expression are available. . . . Some latitude in the manner of expression and the aptness of terms should be permitted even though the claim language is not as precise as the examiner might desire." MPEP 2173.02.

The Federal Circuit has stated that "[t]he definiteness of the language employed must be analyzed--not in a vacuum, but always in light of the teachings of the prior art and of the particular

application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art." *Solomon v. Kimberly-Clark Corp.*, 216 F.3d 1372, 1378 (Fed. Cir. 2000), citing *In re Moore*, 439 F.2d 1232,1235 (CCPA 1971). The MPEP sets forth the same standard:

> "Definiteness of claim language must be analyzed, not in a vacuum, but in light of:
> (A) The content of the particular application disclosure;
> (B) The teachings of the prior art; and
> (C) The claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made." MPEP 2173.02.

The Examiner has failed to meet this standard in assessing the instant claims with respect to definiteness of claim language. As discussed below, the Examiner did not analyze the rejected claims in view of the teachings of the specification or the prior art, or in view of the interpretation by a person of skill in the art, as required for an indefiniteness rejection.

CLAIMS 71 AND 83

The Examiner alleges that "5' purine," as recited in claims 71 and 83, is indefinite because the claims from which these claims depend, claims 63 and 75, respectively, do not provide antecedent basis for this term. The Examiner states that "amended claim 63 requires that the ISS comprises 5' cytosine (C), which is a pyrimidine." The Examiner further states that "amended claim 75 requires that the ISS begins with 5' cytosine (C), which is a pyrimidine." 7/12/05 Office Action, page 12, emphasis added. The Examiner further states that "the term '5'-cytosine guanine-3'' could be any oligonucleotide greater than 8 and less than about 200 nucleotides in length so long the 5' end of the oligonucleotide is cytosine and the 3' end is guanine." 7/12/05 Office Action, page 12.

The Examiner's interpretation of the phrase "comprises 5'-cytosine guanine-3'" is inconsistent with the teachings of the specification and the art, and is inconsistent with the understanding of this phrase by a person of skill in the art.

1. The Examiner has misinterpreted the phrase "ISS comprises 5'-cytosine guanine-3'" in claims 63 and 75, in view of the teaching of the specification.

The Examiner alleges that the language "comprises 5'-cytosine guanine-3'" indicates that "the 5' end of the oligonucleotide is cytosine and the 3' end is guanine." 7/12/05 Office Action, page 12. Applicants disagree with this interpretation of the claim language. The Examiner's interpretation is incorrect and inconsistent with what is taught in the specification.

As described in the specification and well known in the art, a CG (*i.e.*, cytosine guanine) sequence is a necessary feature *within* the sequence of the claimed ISS polynucleotides. The claims use the conventional notation "5'-cytosine guanine-3'" to indicate the order of the cytosine and guanine nucleotides relative to each other within the polynucleotide sequence of the ISS. Further, as is well established in patent law, the term "comprises" is open claim language and "is synonymous with 'including,' 'containing,' or 'characterized by,' . . . and does not exclude additional, unrecited elements." MPEP §2111.03. Thus, the claim language "comprises 5'-cytosine guanine-3'" would be interpreted in accordance with patent law convention as reciting that the ISS *includes* the sequence 5'-cytosine guanine-3', with the possibility of additional nucleotides at the 5' and/or 3' ends of this dinucleotide sequence. This interpretation is borne out by the disclosure of the application, as discussed below.

It is clear from the context provided by the specification that "ISS comprises 5'-cytosine guanine-3'" does not mean that the ISS is required to begin with a cytosine at the 5' end. The specification describes more than 75 examples of ISS for use in the invention, on pages 36-38. Each of these ISS examples includes a CG dinucleotide sequence *within* the sequence of the ISS polynucleotide. Contrary to the Examiner's interpretation of the claim language, *none of the examples in the specification have a C on the 5' end and a G on the 3' end*. The specification states that the claimed ISS "generally comprises the sequence 5'-cytosine, guanine-3', *more particularly comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3'*." Page 36, lines 21-23, emphasis added. Cytosine cannot be on the 5' end of this "particular" example of ISS because it is a pyrimidine, and guanine cannot be on the 3' end of this particular sequence because it is a purine. Thus, the logical interpretation of the language "ISS comprises "5'-cytosine guanine-3'" in view of

this teaching of the specification would be that the ISS *includes* a CG dinucleotide *within* the sequence of the ISS.

The Examiner has failed to analyze the language of claims 63 and 75 in view of "the content of the particular application disclosure" as required by MPEP 2173.02. "[I]t is well-established that the determination whether a claim is invalid as indefinite depends on whether those skilled in the art would understand the scope of the claim when the claim is read in light of the specification." *Solomon v. Kimberly-Clark Corp.*, 216 F.3d 1372, 1378 (Fed. Cir. 2000), citing *Atmel Corp. v. Information Storage Devices*, 198 F.3d 1374, 1378 (Fed. Cir. 1999). With respect to claims 63 and 75, those skilled in the art would understand the scope of the claims as including a CG dinucleotide within the sequence of the ISS in view of the numerous examples provided in the specification that support this interpretation.

2. The Examiner has misinterpreted the phrase "ISS comprises 5'-cytosine guanine-3'" in claims 63 and 75, in view of the teaching in the art.

The teaching in the ISS art contradicts the Examiner's interpretation of the phrase "ISS comprises 5'-cytosine guanine-3'" as requiring a cytosine at the 5' end and a guanine at the 3' end of the ISS. For example, Van Uden et al. (1999) *Journal of Allergy and Clinical Immunology* 104(5): 902-910, cited by the Examiner in the enablement rejection (see above), discusses 36 ISS sequences, *each of which contains a CG sequence within the recited ISS polynucleotide sequence and none of which has a cytosine at the 5' end and a guanine at the 3' end*. Segal et al. (2000) *Journal of Immunology* 164(11): 5683-5688, cited by the Examiner in the enablement rejection (see above), teaches an ISS oligonucleotide that *contains a CG sequence within the recited ISS polynucleotide sequence and does not have a cytosine at the 5' end*. Yamada et al. (2002) *Journal of Immunology* 169(10): 5590-5594, cited by the Examiner in the enablement rejection (see above), teaches two ISS oligonucleotides, *each of which contains a CG sequence within the recited ISS polynucleotide sequence and neither of which has a cytosine at the 5' end and a guanine at the 3' end*.

Thus, the knowledge and teaching in the ISS art do not support the Examiner's interpretation of the language of claims 63 and 75 as requiring a cytosine at the 5' end and a guanine

at the 3' end of the claimed ISS polynucleotide sequences. In view of the teaching in the art of the requirement for a CG dinucleotide within an ISS sequence, a person of skill in the art would interpret the phrase "ISS comprises 5'-cytosine guanine-3'" as indicating that the ISS includes a CG dinucleotide *within* the polynucleotide sequence of the ISS.

3. Claims 63 and 75 provide antecedent basis for claims 71 and 83, respectively.

The Examiner states that "[t]he '5' purine' in claim 71 has no antecedent basis in base claim 63 because 'purine' consists of A or G. However, . . .claim 63 requires that the ISS comprises 5' cytosine (C), which is a pyrimidine." 7/12/05 Office Action, page 12. As discussed above, the phrase "ISS comprises 5'-cytosine guanine-3'" in claim 63 would be interpreted in view of the specification and the art as indicating that a cytosine guanine dinucleotide is included within the ISS polynucleotide sequence. Claim 71 recites that the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. Two 5'-cytosine guanine-3' sequences are comprised (*i.e.*, included) within this sequence. Thus, antecedent basis is provided by claim 63, which requires that a CG sequence be comprised within the ISS sequence. Claim 71 further limits claim 63, because the ISS sequence recited in claim 71 further comprises additional nucleotides (*i.e.*, in addition to the CG sequence for which antecedent basis is provided by claim 63).

The Examiner states that "[t]he '5' purine' in claim 83 has no antecedent basis in base claim 75 because '5'-purine' consists of A or G. However, . . .claim 75 requires that the ISS comprises 5' cytosine (C), which is a pyrimidine." 7/12/05 Office Action, page 12. As discussed above, the phrase "ISS comprises 5'-cytosine guanine-3'" in claim 75 would be interpreted in view of the specification and the art as indicating that a cytosine guanine dinucleotide is included within the ISS polynucleotide sequence. Claim 83 recites that the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. Two 5'-cytosine guanine-3' sequences are comprised (*i.e.*, included) within this sequence. Thus, antecedent basis is provided by claim 75, which requires that a CG sequence be comprised within the ISS sequence. Claim 83 further limits claim 75, because the ISS sequence recited in claim 83 further comprises additional nucleotides (*i.e.*, in addition to the CG sequence for which antecedent basis is provided by claim 75).

4. Other issued U.S. patents contain claims that recite an ISS comprising 5'-cytosine guanine-3', and claims dependent thereon similar to rejected claims 71 and 83.

As discussed above, other issued U.S. patents contain claims that recite an ISS comprising the sequence 5'-cytosine guanine-3', like claims 63 and 75 of the instant application. Each of these patents also contains dependent claims reciting a C,G sequence within a larger ISS polynucleotide sequence, like claims 71 and 83 of the instant application. For example, claim 1 of U.S. Patent No. 6,498,148 recites an ISS comprising the sequence 5'-cytosine-guanine-3', and dependent claim 2 recites "the ISS comprises the sequence 5'-purine-purine-cytosine-guanine-pyrimidine-pyrimidine-3', dependent claim 3 recites "the ISS comprises the sequence 5'-AACGTT-3'," and dependent claims 4-6 recite 41 additional CG-containing sequences, *none of which recites a cytosine at the 5' end and a guanine at the 3' end*. Claim 2 of U.S. Patent No. 6,534,062 recites an ISS comprising the sequence 5'C-G 3', and dependent claim 5 recites an ISS comprising a nucleotide sequence selected from, *inter alia*, CG-containing sequences 5'-purine-purine-cytosine-guanine-pyrimidine-pyrimidine-3' and 5'-purine-TCG-pyrmidine-pyrimidine-3', *neither of which recites a cytosine at the 5' end and a guanine at the 3' end*. Claim 1 of U.S. Patent No. 6,552,006 recites an ISS comprising 5' CpG 3', and dependent claims 3, 4 and 5 recite over 50 CG-containing sequences, *none of which recites a cytosine at the 5' end and a guanine at the 3' end*. Claim 1 of U.S. Patent No. 6,613,751 recites an ISS comprising the sequence 5'-CpG-3', and dependent claims 2, 3, 5, and 7 recite over 50 CG-containing sequences, *none of which recites a cytosine at the 5' end and a guanine at the 3' end*.

Thus, the Office has issued patents with a similar claim configuration to the instant claims, *i.e.*, reciting an ISS comprising 5'-cytosine guanine-3', with claims dependent thereon reciting CG-containing sequences that do not recite a cytosine at the 5' end and a guanine at the 3' end. By virtue of the fact that these claims have issued in U.S. patents, the Office has found claims of such configuration to be definite and in compliance with 35 U.S.C. §112, second paragraph.

CLAIMS 96 AND 100

The Examiner alleges that the term "mammal allergen" in claims 96 and 100 "is ambiguous and indefinite because it is not clear which mammal the allergen belongs." 7/12/05 Office Action, page12.

1. The term "mammal allergen" is clear and definite based on the teachings in the specification.

The specification provides a description of a variety of allergens which may be used in the conjugate molecules of the invention. Table 1 lists a number of known allergens, grouped according to source. For example, "fungal allergens" are allergens produced by fungi and "insect allergens" are those produced by insects. Likewise, "mammal allergens" are those produced by mammals, and examples of such allergens are listed on page 45. Examples of allergens from cats, cows, dogs, horses, and mice are provided.

It is not necessary for Applicants to specify from "which mammal the allergen belongs," as asserted by the Examiner. This term would be clear to a person of skill in the art, in view of the support in the specification describing allergens derived from several different mammalian species. "Determining whether a claim is definite requires an analysis of whether one skilled in the art would understand the bounds of the claim when read in light of the specification. If the claims read in light of the specification reasonably apprise those skilled in the art of the scope of the invention, § 112 demands no more." *Solomon v. Kimberly-Clark Corp.*, 216 F.3d 1372, 1378 (Fed. Cir. 2000), citing *Personalized Media v. International Trade Commission*, 161 F.3d 696, 705 (Fed. Cir. 1998). The Examiner has failed to make a determination concerning the understanding of the term "mammal allergen" by a skilled artisan in view of the teachings of the specification. The description of mammal allergens from several mammalian species in Table 1 would be sufficient to apprise a person of skill in the art regarding the scope of the claimed invention (*i.e.*, an allergen derived from a mammalian species). Applicants submit that claims 96 and 100 are clear and definite, in accordance with 35 U.S.C. §112, second paragraph.

2. Other issued U.S. patents contain claims that recite the term "mammalian antigen"

Applicants note that other issued U.S. patents contain claims that recite the term "mammalian antigen," indicating that the Office has found such a term to be definite and in compliance with 35 U.S.C. §112, second paragraph. U.S. Patent No. 6,793,923 contains claims directed to a vaccine composition comprising an antigen encapsulated in liposomes. Dependent claim 8 recites that "the antigen is a viral, bacterial, protozoal or *mammalian antigen*." The specification does not provide a definition for this term other than stating that an antigen may be of mammalian origin (col. 4, lines 48-49). U.S. Patent No. 6,339,068 contains claims directed to methods for producing ISS constructs comprising at least one CpG-S motif and a nucleic acid encoding an antigen, wherein the antigen is selected from the group consisting of, *inter alia*, a *mammalian antigen*. No definition for "mammalian antigen" is provided in the specification. Thus, the Office has found claims reciting this term to be definite and in compliance with 35 U.S.C. §112, second paragraph, without requiring a recitation of "which mammal the allergen belongs," as asserted by the Examiner in the instant case (7/12/05 Office Action, page 12).

VIII. CLAIMS APPENDIX

A copy of the claims involved in the present appeal is attached hereto as Appendix A. As indicated above, the claims in Appendix A do not include the amendments filed on January 12, 2006.

IX. EVIDENCE APPENDIX

Evidence entered by the Examiner and relied upon by Applicants in the appeal, are attached hereto as Appendix B.

X. RELATED PROCEEDINGS APPENDIX

No related proceedings are referenced in II. above. Hence, no Appendix is included.

Dated: August 14, 2006

Respectfully submitted,

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 09/713,136

63. A population of conjugate molecules, said conjugate molecules comprising an allergen and a polynucleotide comprising an immunostimulatory sequence (ISS), wherein said ISS comprises 5'-cytosine guanine-3', wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length and wherein the extent of conjugation in the population provides an average of at least 5.5 ISS-containing polynucleotides per allergen molecule.

71. The population of claim 63, wherein said ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

72. The population of claim 71, wherein said ISS comprises a sequence selected from the group consisting of GACGCTCG, GACGTCCG, GACGCCCG, GACGTTCG, AGCGCTCG, AGCGTTCG, AGCGTCCG, AGCGCCCG, AACGTCCG, AACGCCCG, AACGTTCG, AACGCTCG, GGCGTTCG, GGCGCTCG, GGCGTCCG, and GGCGCCCG.

74. A composition comprising the population of claim 63 in a pharmaceutically acceptable excipient.

75. A population of conjugate molecules, said conjugate molecules comprising an allergen and a polynucleotide comprising an immunostimulatory sequence (ISS), wherein said ISS comprises 5'-cytosine guanine-3', wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length and wherein the extent of conjugation in the population provides a ratio of (i) average mass of ISS-containing polynucleotide to (ii) average mass of allergen of at least about 45 to about 40.

83. The population of claim 75, wherein said ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

84. The population of claim 83, wherein said ISS comprises a sequence selected from the group consisting of GACGCTCG, GACGTCCG, GACGCCCG, GACGTTCG,

AGCGCTCG, AGCGTTCG, AGCGTCCG, AGCGCCCG, AACGTCCG, AACGCCCG, AACGTTCG, AACGCTCG, GGCGTTCG, GGCGCTCG, GGCGTCCG, and GGCGCCCG.

86. A composition comprising the population of claim 75 in a pharmaceutically acceptable excipient.

95. The population according claim 63, wherein the allergen is Amb a 1.

96. The population according to claim 63, wherein the allergen is selected from the group consisting of a pollen allergen, an insect allergen, a mammal allergen, a nut allergen, a crustacean allergen and a fungal allergen.

97. The population according to claim 63, wherein the allergen is selected from the group consisting of a ragweed allergen, a grass allergen, a birch allergen, a cedar allergen, a juniper allergen, a dust mite allergen, a cockroach allergen, a cat allergen, a dog allergen, a peanut allergen, a wheat allergen and a latex allergen.

99. The population according claim 75, wherein the allergen is Amb a 1.

100. The population according to claim 75, wherein the allergen is selected from the group consisting of a pollen allergen, an insect allergen, a mammal allergen, a nut allergen, a crustacean allergen and a fungal allergen.

101. The population according to claim 75, wherein the allergen is selected from the group consisting of a ragweed allergen, a grass allergen, a birch allergen, a cedar allergen, a juniper allergen, a dust mite allergen, a cockroach allergen, a cat allergen, a dog allergen, a peanut allergen, a wheat allergen and a latex allergen.

106. The population according to claim 63, wherein the allergen is a polypeptide.

107. The population according to claim 75, wherein the allergen is a polypeptide.

108. A population of conjugate molecules made by the process comprising: combining a polynucleotide comprising an immunostimulatory sequence (ISS) and allergen at a ratio of about 17 molar equivalents of the polynucleotide to about 1 molar equivalent of the allergen whereby conjugate molecules comprising the polynucleotide and allergen are formed, wherein the polynucleotide is greater than 8 and less than about 200 nucleotides in length and wherein the ISS comprises 5'-cytosine guanine-3'.

APPENDIX B

Evidence Appendix

Tab	Reference
1.	Stryer et al., *Biochemistry*, 3rd edition, W.H. Freeman and Co., New York, 1988, pages 31-33.
2.	Ngo et al., *The Protein Folding Problem and Tertiary Structure Prediction*, K. Merz, Jr. and S. LeGrand, editors, Birkhauser, Boston, 1994, pages 491-495.
3.	Chatel et al. (2003) *Allergy* 58:641-647.
4.	Van Uden et al. (1999) *Allergy and Clinical Immunology* 104(5):902-907.
5.	Segal et al. (2000) *Journal of Immunology* 164(11):5683-5688.
6.	Yamada et al. (2002) *Journal of Immunology* 169(10):5590-5594.
7.	U.S. Patent No. 6,498,148
8.	U.S. Patent No. 6,534,062
9.	U.S. Patent No. 6,552,006
10.	U.S. Patent No. 6,613,751
11.	U.S. Patent No. 6,793,923
12.	U.S. Patent No. 6,339,068

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Four levels of structure are frequently cited in discussions of protein architecture. *Primary structure* is the amino acid sequence and the location of disulfides, if there are any. The primary structure is thus a complete description of the covalent connections of a protein. *Secondary structure* refers to the spatial arrangement of amino acid residues that are near one another in the linear sequence. Some of these steric relationships are of a regular kind, giving rise to a periodic structure. The α helix, β pleated sheet, and collagen helix are elements of secondary structure. *Tertiary structure* refers to the spatial arrangement of amino acid residues that are far apart in the linear sequence. The dividing line between secondary and tertiary structure is a matter of taste. Proteins containing more than one polypeptide chain exhibit an additional level of structural organization. Each polypeptide chain in such a protein is called a subunit. *Quaternary structure* refers to the spatial arrangement of such subunits and the nature of their contacts (Figure 2-40). The constituent chains of a multisubunit protein can be identical or different. For example, immunoglobulin G, the major antibody molecule in plasma, consists of two L chains and two H chains. The spherical shell of tomato bushy stunt virus, a plant pathogen, is formed from 180 identical coat protein molecules. The interfaces between subunits are often functionally significant. For example, in hemoglobin (consisting of four chains), the subunit interfaces participate in transmitting information between binding sites for O_2, CO_2, and H^+. In antibody molecules, the combining site for antigen is formed by segments of two different kinds of chains.

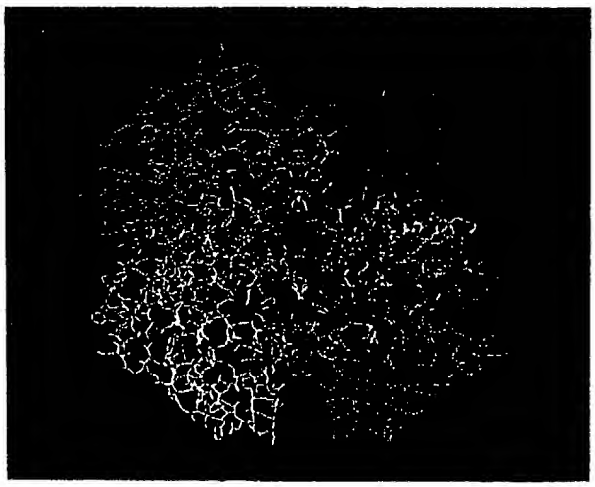


Figure 2-40
Three-dimensional structure of hemoglobin. The four subunits are shown in different colors. Each contains an oxygen-binding heme group (red).

Recent studies of protein conformation, function, and evolution have revealed the importance of two additional levels of organization. *Supersecondary structure* refers to clusters of secondary structure. For example, a β strand separated from another β strand by an α helix is found in many proteins; this motif is called a $\beta\alpha\beta$ unit. It is fruitful to regard supersecondary structures as intermediates between secondary and tertiary structure. Some polypeptide chains fold into two or more compact regions that may be joined by a flexible segment of polypeptide chain, rather like pearls on a string. These compact globular units, called *domains*, range in size from about 100 to 400 amino acid residues. For example, a 25-kd L chain of an antibody is folded into two domains (Figure 2-41). Indeed, these domains resemble one another, which sug-

Figure 2-41
The light (L) chain of an antibody molecule consists of two distinct domains.

gests that they arose by duplication of a primordial gene. An important principle has emerged from analyses of genes and proteins in higher eucaryotes: *protein domains are often encoded by distinct parts of genes called exons* (p. 112). In our explorations of genes and proteins, exons and domains will often be at the focal point.

AMINO ACID SEQUENCE SPECIFIES THREE-DIMENSIONAL STRUCTURE

Insight into the relation between the amino acid sequence of a protein and its conformation came from the work of Christian Anfinsen on ribonuclease. As mentioned earlier, ribonuclease is a single polypeptide chain consisting of 124 amino acid residues (Figure 2-42). Its four disul-

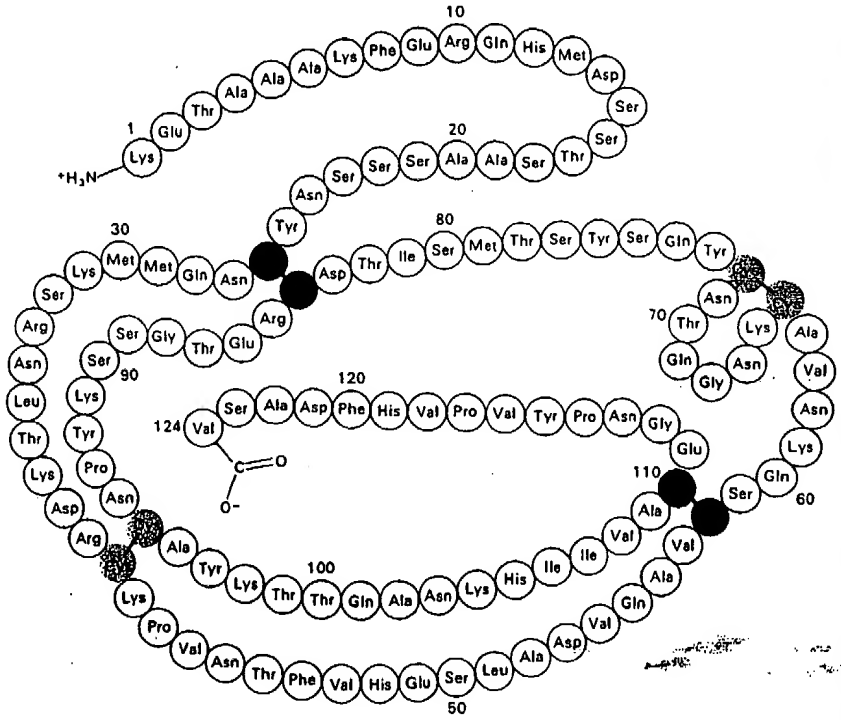
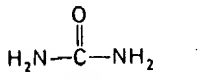
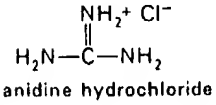


Figure 2-42
Amino acid sequence of bovine ribonuclease. The four disulfide bonds are shown in color. [After C. H. W. Hirs, S. Moore, and W. H. Stein. *J. Biol. Chem.* 235(1960):633.]

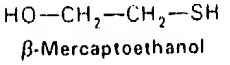
fide bonds can be cleaved reversibly by reducing them with a reagent such as *β-mercaptoethanol*, which forms mixed disulfides with cysteine side chains (Figure 2-43). In the presence of a large excess of β-mercaptoethanol, the mixed disulfides also are reduced, so that the final product is a protein in which the disulfides (cystines) are fully converted into sulfhydryls (cysteines). However, it was found that ribonuclease at 37°C and pH 7 cannot be readily reduced by β-mercaptoethanol unless the protein is partly unfolded by agents such as *urea* or *guanidine hydrochloride*. Although the mechanism of action of



Urea



Guanidine hydrochloride



β-Mercaptoethanol

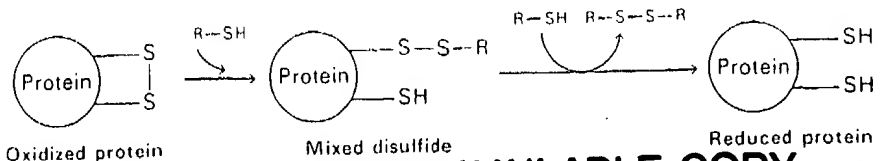


Figure 2-43
Reduction of the disulfide bonds in a protein by an excess of a sulfhydryl reagent such as β-mercaptoethanol.

these agents is not fully understood, it is evident that they disrupt noncovalent interactions. Most polypeptide chains devoid of cross-links assume a *random-coil conformation* in 8 M urea or 6 M guanidine HCl, as evidenced by physical properties such as viscosity and optical rotatory spectra. When ribonuclease was treated with β-mercaptoethanol in 8 M urea, the product was a fully reduced, randomly coiled polypeptide chain *devoid of enzymatic activity*. In other words, ribonuclease was *denatured* by this treatment (Figure 2-44).

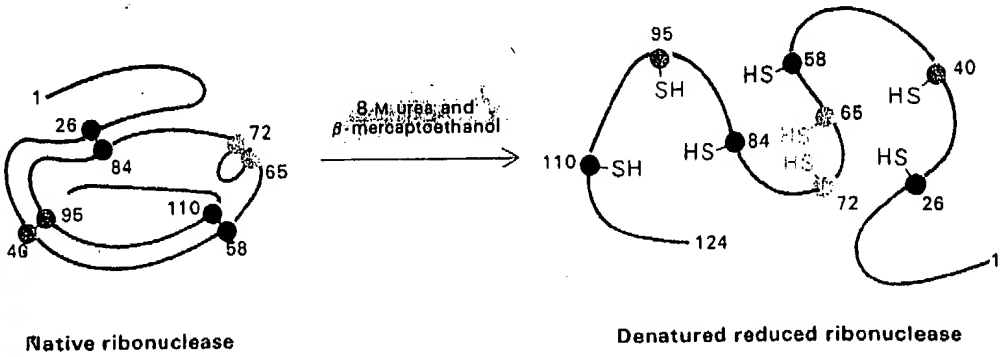


Figure 2-44
Reduction and denaturation of ribonuclease.

Anfinsen then made the critical observation that the denatured ribonuclease, freed of urea and β-mercaptoethanol by dialysis, slowly regained enzymatic activity. He immediately perceived the significance of this chance finding: the sulfhydryls of the denatured enzyme became oxidized by air and the enzyme spontaneously refolded into a catalytically active form. Detailed studies then showed that nearly all of the original enzymatic activity was regained if the sulfhydryls were oxidized under suitable conditions (Figure 2-45). All of the measured physical and chemical properties of the refolded enzyme were virtually identical with those of the native enzyme. These experiments showed that *the information needed to specify the complex three-dimensional structure of ribonuclease is contained in its amino acid sequence*. Subsequent studies of other proteins have established the generality of this central principle of molecular biology: *sequence specifies conformation*.

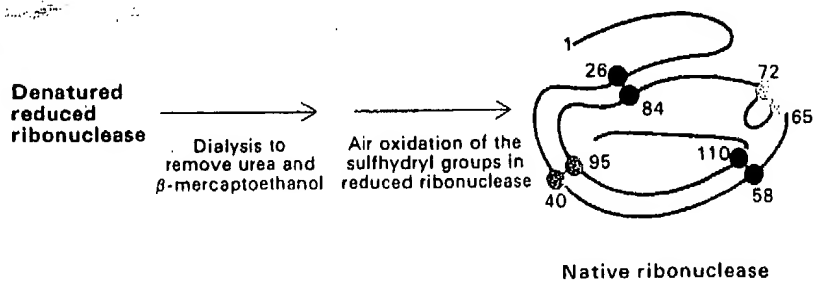


Figure 2-45
Renaturation of ribonuclease.

A quite different result was obtained when reduced ribonuclease was reoxidized while it was still in 8 M urea. This preparation was then dialyzed to remove the urea. Ribonuclease reoxidized in this way had only 1% of the enzymatic activity of the native protein. Why was the outcome of this experiment different from the one in which reduced ribonuclease was reoxidized in a solution free of urea? The reason is that wrong disulfide pairings were formed when the random-coil form of the reduced molecule was reoxidized. There are 105 different ways

14

Computational Complexity, Protein Structure Prediction, and the Levinthal Paradox

J. Thomas Ngo, Joe Marks, and Martin Karplus

1. Perspectives and Overview

A protein molecule is a covalent chain of amino acid residues. Although it is topologically linear, in physiological conditions it folds into a unique (though flexible) three-dimensional structure. This structure, which has been determined by x-ray crystallography and nuclear magnetic resonance for many proteins (Bernstein et al., 1977; Abola et al., 1987), is referred to as the native structure. As demonstrated by the experiments of Anfinsen and co-workers (Anfinsen et al., 1961; Anfinsen, 1973), at least some protein molecules, when denatured (unfolded) by disrupting conditions in their environment (such as acidity or high temperature) can spontaneously refold to their native structures when proper physiological conditions are restored. Thus, all of the information necessary to determine the native structure can be contained in the amino acid sequence.

From this observation, it is reasonable to suppose that the native fold of a protein can be predicted *computationally* using information only about its chemical composition. In particular, it should be possible to write down a mathematical problem that, when solved, gives the native conformation of the protein. This procedure would be self-contained, in the sense that no additional information about the biology of protein synthesis would be required. Further, it is reasonable to hope that this procedure could be accomplished without requiring an astronomical amount of computer resources, given the observation that polypeptide chains do fold to their

The Protein Folding Problem and Tertiary Structure Prediction
K. Merz, Jr. and S. Le Grand, Editors

which depends steeply on the energy gap U. Given the assumptions that $N = 100$ and $n = 2$, it was found that in the limit $U \to 0$, the first-passage time is nearly 10^{30} years. However, a modest change to the value of U, say $U = 2kT$, lowers the first-passage time to under one second. (The base of the exponential, $1 + n\exp(-U/kT)$, is equal to 3 when $U = 0$, but 1.27 when $U = 2kT$.)

The analysis of Zwanzig et al. resolves a form of the Levinthal paradox in which the absence of clues about the form of the native state is the sole basis for expecting exponential-time folding. However, it does not resolve the form of the paradox based on computational complexity, since the optimization problem implied by the underlying model can be solved trivially in linear time. The reason for the tractability of the underlying model is the lack of long-range interactions, which are critical to rendering PSP NP-hard (Ngo and Marks, 1992), and essential for cooperativity (Karplus and Shakhnovich, 1992).

6. Future Work

It is not known whether there exists an efficient algorithm for predicting the structure of a given protein from its amino acid sequence alone. Decades of research have failed to produce such an algorithm, yet Nature seems to solve the problem. Proteins do fold! The "direct" approach to structure prediction, that of directly simulating the folding process, is not yet possible because contemporary hardware falls eight to nine orders of magnitude short of the task. However, while this difference is large, it is not astronomical. Would this "direct" approach constitute an efficient and correct algorithm for protein-structure prediction? Too little is known about protein folding, and about the future of computing technology, to be able to answer this question at this time.

The results reviewed here (Section 3) do not completely rule out the existence of a protein-structure prediction algorithm that is both efficient and correct, in the precise senses of those words used throughout this chapter. In particular, it remains formally possible that there is a restricted form of PSP that is efficiently solvable, but subsumes protein-structure prediction. How can this possibility be investigated?

A standard strategy in the analysis of any NP-hard problem is to examine restricted forms of the problem systematically, classifying each as tractable or NP-hard, and thereby exposing the sources of the complexity. Barahona's results with Ising spin-glass models, which were described briefly in Section 4, are exemplary of this approach. While the particular

restrictions chosen by Barahona for spin glasses (reduction of dimensionality and removal of the magnetic field) are not suitable for protein-structure prediction, the overall strategy of examining restricted forms is appropriate. Some restricted form of PSP in which compactness plays a critical role is a candidate for this type of analysis (Section 4.6).

The approach of considering restricted forms has worked well for dozens of important problems that are relatively "clean" and abstract (Garey and Johnson, 1979), but it may be difficult to pursue in the case of protein-structure prediction. In the former case, the problem shown to be NP-hard is usually as general as would actually be required in practice. In the latter case, what is desired is not an algorithm that can handle all possible instances of PSP (Section 3), but merely one that works for proteins. Thus, the fact that PSP is a generalization of protein-structure prediction makes the result that PSP is NP-hard less limiting than it could be.

Ideally, one would like to demonstrate the NP-hardness of a problem that is more *specific*, not more general, than protein-structure prediction, because that would automatically prove the NP-hardness of protein-structure prediction itself. This would entail finding an efficient transformation from some existing NP-complete problem that generates instances of PSP that are proteins by every conceivable criterion.[38] It is difficult to see how such a transformation might proceed.[39]

An alternative approach that may be nearly as instructive is to use the currently available result regarding PSP as a baseline in a continuing comparative analysis—to find restricted forms of PSP that are NP-hard but as specialized as possible, and to find others that are tractable but as general as possible. The motivations for pursuing this methodology are both practical and theoretical:

- Every NP-hardness result permits us to know in advance that a certain group of algorithms is likely to fail, and is therefore not worth pursuing (Section 4).
- Conversely, every NP-hardness result helps identify a source of complexity in protein-structure prediction, and therefore what must be stripped away from the problem before it is reasonable to attempt efficient solution.

  The work of Finkelstein and Reva (1992) is a good example; an approach to structure prediction with a guaranteed polynomial time bound was developed. The critical assumption behind the algorithm is that only nonbonded interactions between nearest neighbors along the chain are significant. Because of this assumption, the algorithm cannot solve all instances of PSP, but instead is restricted to instances in

which only nonbonded interactions between nearest neighbors along the chain are nonzero.[40] This violates the requirements of the reduction from Partition to PSP, in which nonbonded interactions between sites distant from each other along the chain are essential. Thus, the problem is similar in character to that examined by Zwanzig et al. (Section 5.3). While the Finkelstein–Reva algorithm was not inspired by an NP-hardness result, the underlying strategy is similar to how NP-hardness results might be used; they removed from the problem what they observed to be a source of complexity. However, in this case, removing the source of complexity led to a problem different from that posed by protein folding, in which long-range interactions play an essential role.

- The NP-hardness of PSP serves as the premise for a reformulation of the Levinthal paradox (Section 5), whose conventional form is based on a model of folding that is in conflict with known experimental results. A motivation for pursuing an analysis of the computational complexity of protein-structure prediction is to assist in the constructive role of the Levinthal paradox—to help focus attention on the key questions in protein folding.

  A small number of reasonably well-defined potential resolutions to the computational-complexity form of the Levinthal paradox were listed in Section 5. One of the possible resolutions is that protein-structure prediction is tractable. NP-hardness results with restricted forms of PSP would make that possible resolution less likely, thus lending credence to the alternatives.

  Attempts to resolve the Levinthal paradox, which play a valid and useful role in helping to understand how proteins fold, can lead to confusion because the premises of the original form of the paradox are not well formulated. In particular, one such proposed resolution (Zwanzig et al., 1992) can be shown unequivocally not to resolve the computational complexity form of the paradox, and in related arguments (Karplus and Shakhnovich, 1992) has been shown to lead to physically incorrect consequences (Section 5.3). For the paradox to be meaningful, it must be "falsifiable"—it must be possible to know when the paradox has been resolved.

In addition to restricted forms of PSP, it would be useful to know the computational complexity of other tasks in structure prediction that appear easier than the general problem, but whose complexities are none the less uncertain.

The task of computing side-chain conformations given full knowledge of a protein's backbone conformation is one such problem. Case studies using simulated annealing (Lee and Subbiah, 1991) have suggested that packing effects may suffice to determine, in part, the side-chain conformations in a protein's core. The computational complexity of this packing problem is unknown. Because only short-range effects are present, the graph of possible side-chain–side-chain interactions can be known in advance, is sparse, and consists of vertices of low degree. Previous experience—for instance, with Ising spin-glass models (Barahona, 1982), graph colorability (Garey and Johnson, 1979, p. 191) and cartographic labeling (Formann and Wagner, 1991; Marks and Shieber, 1991)—illustrates that such neighborhood interactions can, on their own, give rise to NP-hardness. On the other hand, many problems that contain such neighborhood interactions are tractable if restrictions can be placed on the nature of the graph (Garey and Johnson, 1979), suggesting that the problem of finding a mutually acceptable set of side-chain conformations for a protein could be tractable. (One currently known algorithm for predicting side-chain conformations based on backbone positions achieves 70% to 80% accuracy for χ_1 and χ_2 angles [Dunbrack and Karplus, 1993].) Not knowing the computational complexity of side-chain structure prediction leaves the algorithm developer in the quandary of not knowing whether inexact methods are truly necessary, given the possible existence of a superior exact algorithm.

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NOTES

[1] The Thermodynamic Hypothesis states that a protein's native fold is the configuration of globally minimal free energy. However, it is generally assumed that a protein's states of lowest free energy are similar enough in entropy to justify the use of potential energies instead of free energies as a computational convenience; potential energies are much faster and more straightforward to compute.

[2] For example, if only nonbonded interactions between nearest neighbors along the chain are significant, the global minimum structure can be predicted efficiently (Finkelstein and Reva, 1992).

[3] The term *combinatorial optimization* is normally reserved for problems in which the solution space is discrete. Throughout this chapter we use the term to refer

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Original article

Various factors (allergen nature, mouse strain, CpG/recombinant protein expressed) influence the immune response elicited by genetic immunization

Background: Genetic immunization is a very promising therapeutic approach for allergy treatment. In the present study we investigate the influence of the nature of the allergen, the mouse strain, and the relative amount of CpG to expressed recombinant protein on immune responses using two major peanut allergens, Ara h 1 and Ara h 4.
Methods: The cDNA of Ara h 1 and of an isoform of Ara h 4 were cloned and inserted in pcDNA3. Antigen specific IgG1, IgG2a and IgE were followed after genetic immunization with 100 μg of these clones in mouse strain SKH-Hr1 or BALB/c and with 1 μg of the clones + 99 blank plasmid in SKH-Hr1.
Results: Genetic immunization in SKH-Hr1 with Ara h 1 elicited a classical Th1 type response, but Ara h 4 elicited a mixed Th1/Th2 response with high IgG1 and even IgE in some mice. In BALB/c both plasmids produced a high IgG1 level. Decreasing the amount of plasmid injected did not change the immune response profile. However, increasing the amount of CpG administered relative to the recombinant Ara h 4 protein expressed reversed the Th1/Th2 response pattern in SKH-Hr1 mice.
Conclusions: Immune responses after genetic immunization are strongly influenced by the nature of the allergen, the mouse strain, and the ratio of CpG to recombinant protein expressed.

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Key words: allergy; genetic immunization; peanuts.

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Food allergy poses a considerable public health problem especially in children because of the potential severity of allergic hypersensitivity. In the US, food allergies are the most common cause of anaphylaxis treated in hospital emergency rooms (1). Peanuts and tree nuts together are the leading cause of fatal and near-fatal reactions (2). At the present time there are no safe and effective therapies for food allergies except avoidance. Unfortunately, accidental ingestion of peanut containing food is not rare and results in many deaths each year (3).

Intramuscular or intradermal injection of plasmid DNA encoding antigens, called genetic immunization, induces a strong T helper cell type 1 (Th1) response in contrast to the Th2-type response after conventional immunization with protein (4). The major reason for this Th1-type response is thought to be the presence of immunostimulatory sequences (ISS) in the plasmid DNA vector used for immunization (5). These ISS contain unmethylated CpG motifs flanked by two 5′ purines and two 3′ pyrymidines, which bind to toll-like receptor 9 on macrophages and dentritic cells (6), thereby stimulating the production of Th1-biasing cytokines (7).

In this paper using DNA plasmids encoding for peanut allergen Ara h 1 (pAra h 1) and an isoform of Ara h 4 and Ara h 3 (pAra h 4), we found that the Th1/Th2 bias of the resulting immune response after genetic immunization was influenced by multiple factors, including the nature of the allergen protein, the mouse strain, and the relative ratio of the quantity of ISS to the recombinant protein expressed. Genetic immunization in SKH-Hr1 mice with pAra h 1 resulted in the expected Th1 biased response with a high level of IgG2a and no detectable IgE. In contrast, immunization with pAra h 4 in the same mouse strain resulted in a Th2 bias with a high level of IgG1 and even the presence of IgE. In BALB/c, administration of both plasmids elicits a strong IgG1 response. The Th2 immune response profile for pAra h 4 in SKH-Hr1 mice was reversed by increasing the amount of administered CpG relative to the expressed recombinant protein.

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Material and methods

DNA purification from λgt11 cDNA library

A λgt11 cDNA library constructed with RNA from developing seeds of F78-1339 (*A. hypogaea*) as described (8) was a kind gift of Albert Abbott (Clemson University, Clemson, SC, USA). The titer of the cDNA library, $1.8/10^8$ pfu/ml was obtained as described (9). Fifty thousand pfu were diluted in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCl pH 7.5, 0.01% gelatin) and added to 500 µl of *E.Coli* MRA previously grown until $OD_{600} = 0.5$ and resuspended in 10 mM $MgSO_4$. After 15 min, at 37°C, we added 7.5 ml of LB M/M Top agarose: (LB broth, maltose 0.2%, $MgSO_4$ 10 mM, high melting point agarose 0.4%, low melting point agarose 0.3%) and plated on 150 mm NZY Agarose plates (yeast extract 0.5%, N-Z-Amine A 1%, Casaminoacids 0.1%, NaCl 100 mM, $MgSO_4$ 8 mM, high melting point agarose 0.7%). After an overnight (ON) incubation, at 37°C, 5 ml SM buffer was added on each plate. Plates were shaken at room temperature (room temperature) for 1 h. Then, we added 2 ml chloroform for each plate and incubated it for 1 h at 37°C with shaking. The slurry was collected and then centrifuged at 15 000 *g* for 15 min. The supernatant was loaded on gradient 40/5% glycerol in TMG (10 mM Tris–HCl pH 7.5, $MgSO_4$ 5 mM, gelatin 0.01%) and then centrifuged at 28 000 rpm for 2 h. For each 10 plates, the pellet was resuspended in 5 ml TMG buffer. DNAse I was added to a final concentration of 1 µg/ml, and RNAse was added to a final concentration of 5 µg/ml. The suspension was then incubated at 37°C for 30 min. Enzymes were inactivated by the addition of EDTA (final concentration 20 mM) and SDS (final concentration 0.5%) with a 15 min incubation at 70°C. Then, proteinase K was added (final concentration 50 µg/ml), and the solution was incubated at 60°C for 1 h, followed by inactivation at 70°C for 1 h. The solution was extracted with phenol/chloroform until the interface was clear, and the DNA was precipitated by ethanol.

Amplification and cloning of Ara h 1 and Ara h 4

Amplification by PCR was done on purified DNA from λgt11 cDNA using the Expand High Fidelity PCR System (Boehringer). Primers used were pair H1F1 (ATG AGA GGG AGG GTT TC TCC A) and H1R1 (TCA GTT AAA AGC CTT CAA AAT) priming, respectively, at the N- and C-terminus of Ara h 1 (Genbank L34402) and pair H4F1 (ATG GCT AAG CTT CTT GAG CTT TCT TTT TGC) and H4R1 (TTA AGC CAC ACG CCT CGG AGA CTG CTG AAA) priming respectively at N- and C-terminus of Ara h 4 (Genbank AF086821). The typical program used was 94°C for 10 min (one cycle); then 94°C for 1 min, 55°C for 1 min, 72°C for 1 min (25 cycles) and finally 72°C for 10 min (one cycle). Cloning was done in pcDNA3.1/V5-His using pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen) as described by the supplier. Briefly 2 µl of each amplification reaction was directly added to 1 µl of pcDNA3.1/V5-His TOPO vector, 1 µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and 2 µl of H_2O for 30 min at room temperature. Then, 2 µl of the cloning reaction was transformed in TOP10 chemically competent cells provided by the supplier. Transformed cells were spread on ampicillin (50 µg/ml) plates, and incubated overnight at 37°C.

Positive clones were first selected for the presence of an insert of the right size by PCR on 20 isolated colonies using primer H1F1 and H1R1 or H4F1 and H4R1. Positive clones after the first selection were tested for an insert in the correct orientation for expression by PCR using T7F promoter (5′ of the cloning site) primer and H1R1 or H4R1. Positive clones after the second selection were confirmed by another PCR using primers H1F1 or H4F1 and BGH Reverse primer (3′ of the cloning site).

Selected clones were amplified on selective medium and plasmids were purified (Plasmid Mini Kit, Quiagen), and then sequenced (Lone Star Labs Inc., Houston, TX).

Immunization

All animal experiments were approved by the Institutional Review Board for Animal Studies (Baylor College of Medicine). Groups of mice were injected intradermally in the skin 1 cm from the base of the tail (10) with a total of 100 µg plasmid DNA in two separate 25 µl PBS injections, except as otherwise specified. Plasmids were prepared using the Endofree Plasmid Giga Kit (Qiagen, Valencia, CA). Sequential serum samples were obtained by tail incision bleed and were assayed fresh or were stored frozen (−20°C) until assayed. The IgG subclass response patterns were analyzed by the Mann–Whitney rank sum test for each group of mice.

Immunoassays for the detection of specific IgG1, IgG2a and IgE anti-peanut extract

Ninety-six-well plates were coated passively with 1 µg/ml of peanut extract diluted in phosphate buffer 50 mM, pH 7.4, for 18 h at room temperature. After washing, the plates were saturated with phosphate buffer 50 mM, pH 7.4, NaCl 0.15 M, BSA 0.1% (EIA buffer), for 18 h at room temperature. Sera were serially diluted in EIA buffer and incubated overnight at 4°C, in 96-well plates previously coated with peanut extract. The plates were washed five times with PBS-Tween (0.1%). Bound Abs were detected with HRP-conjugated goat anti-mouse IgG1 (SBA, Birmingham, AL), or rat anti-mouse IgG2a (Serotec, England) or rat anti-mouse IgE (clone LO-ME-3) (Serotec, England). Second antibodies were added for 2 h at room temperature. After five washes with PBS-Tween (0.1%) reactions were developed with the TMB liquid substrate system for ELISA (Sigma, St Louis, MI, USA). Reactions were stopped by addition of 1 volume of sulfuric acid 2N. The resulting yellow end product was read at 450 nm with an SLT microplate reader (TELAC Inc., Research Triangle Park, NC). The background was determined using preimmune sera. Mice sera immunized with peanut extract in alum were used as a positive control. Naive mice sera were used as a negative control.

Results

Cloning and characterization of Ara h 1 and Ara h 4

Ara h 1 and Ara h 4 were cloned by PCR using a polymerase with proof reading activity and N- and C-terminal oligonucleotides. Nucleotide sequences were deduced from the published sequence of Ara h 1 (clone p41b, Genbank L34402) (11) and Ara h 4 (Genbank AF086821) (12). Ara h3 (Genbank AF093541) (13) and Ara h 4 are two isoforms that are members of the 11S protein storage family, 92% homologous in amino acids (Fig. 1). We chose to use the Ara h 4 sequence because the cDNA was complete. Each amplification produced only one band at the predicted size (data not shown). The amplification products were cloned in the pCDNA3 vector and both sense and anti-sense strands were sequenced. We found one type of Ara h 1 clone, 100%

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Ara h 412 MAKLLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPNN
Ara h 3   ----------------------RQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPNN
Ara h 4   MAKLLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPNN
                                 ************************************

Ara h 412 QEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPG PSTYEEPAQQGR
Ara h 3   QEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPG PRHYEEPHTQGR
Ara h 4   QEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFPG PSTYEEPAQQGR
          ************************************************  ****  ***

Ara h 412 RSQSQRPPRRLQGEDQSQQQQDSHQKVHRFDEGDLIAVPTGVAFWLYNDHDTDVVAVSLT
Ara h 3   RSQSQRPPRRLQGEDQSQQQRDSHQKVHRFDEGDLIAVPTGVAFWLYNDHDTDVVAVSLT
Ara h 4   RYQSQRPPRRLQEEDQSQQQQDSHQKVHRFNEGDLIAVPTGVAFWLYNDHDTDVVAVSLT
          * ********** ******* ********* ******************************

Ara h 412 DTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQSRQSRRRSLPYSPYSPQSQPRQEEREFS
Ara h 3   DTNNNDNQLDQFPRRFNLAGNTEQEFLRYQQQSRQSRRRSLPYSPYSPQSQPRQEEREFS
Ara h 4   DTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQSRQSRRRSLPYSPYSPHSRPRREEREFR
          ******************** ************************** * ** *****

Ara h 412 PRGQHSRRERAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGENESEEEGAIVT
Ara h 3   PRGQHSRRERAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGETESEEEGAIVT
Ara h 4   PRGQHSRRERAGQEEEDEGGNIFSGFTPEFLEQAFQVDDRQIVQNLWGENESEEEGAIVT
          **************** ***************************** ** **********

Ara h 412 VRGGLRILSPDRKRGADEEEEYDEDEYEYDEEDRRRGRGSRGRGNGIEETI TASVKKNI
Ara h 3   VRGGLRILSPDRKRRADEEEEYDEDEYEYDEEDRRRGRGSRGRGNGIEETI TASAKKNI
Ara h 4   VRGGLRILSPDGTRGADEEEEYDEDQYEYHEQDGRRGRGSRGGGNGIEETI TACVKKNI
          ***********  * ********** *** * * ******** ***********  ****

Ara h 412 GRNRSPDIYNPQAGSLKTANDLNLLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYAL
Ara h 3   GRNRSPDIYNPQAGSLKTANDLNLLILRWLGPSAEYGNLYRNALFVAHYNTNAHSIIYRL
Ara h 4   GGNRSPHIYDPQRWFTQNCHDLNLLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYAL
          ************        *********** ************** *********** *

Ara h 412 RGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSDNFEYVAFKTDSRPSIANL
Ara h 3   RGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEYVAFKTDSRPSIANL
Ara h 4   RGRAHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEYVAFKTDSRPSIANF
          ****************************************** *****************

Ara h 412 AGENSVIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPSQQSPRRVA
Ara h 3   AGENSVIDNLPEEVVANSYGLQREQARQLKNNNPFKFFVPPSQQSPRAVA
Ara h 4   AGENSFIDNLPEEVVANSYGLPREQARQLKNNNPFKFFVPPFQQSPRAVA
          ***** *************** ******************* ***** **
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Figure 1. Sequence comparison between allergens Ara h 3, Ara h 4 and clone Ara h 412. Alignment has been done using CLUSTAL W (26). Homology between three sequences is indicated by *. Linear IgE binding epitopes of Ara h 3 (13) are underlined. Essential amino acids for IgE binding in each epitope are in italics. The proteolysis sequence consensus is in bold. Cysteine residues implicated in conserved disulfide bridge are in gray.

homologous to clone p41b of Ara h 1 and 97% homologous in nucleotide or amino acids to clone p17 (Genbank L38853). None of our clones were 100% homologous to the published isoform sequences for Ara h 4 or Ara h 3, and the clone we selected for further use, Ara h 412, is another isoform of the 11S family, 97% homologous in amino acids to Ara h 3 and 94% to Ara h 4 (Fig. 1). The plasmids used for the genetic immunization studies were named, respectively, pAra h 1 and pAra h 4.

ID Injection of 100 μg of pAra h 1 or pAra h 4 in SKH-HR1 mice

Genetic immunizations used 100 μg of either pAra h 1 or pAra h 4 injected intradermally in the tail of SKH-HR1 mice ($n = 4$ for pAra h 1, $n = 5$ for pAra h 4). After 4 weeks, a second injection, or boost, of 100 μg was done. The mice were bled at 4 weeks, before the boost injection, and again at 8 weeks, 4 weeks after the boost injection. The IgG1 and IgG2a responses were monitored in the sera by ELISA using plates coated with peanut extract or purified Ara h 1 protein. At 8 weeks after the first injection, both groups of mice showed IgG1 and IgG2a responses (Fig. 2). In the response to pAra h 1, the IgG2a responses were consistently higher than in the IgG1 responses ($P < 0.026$, Mann–Whitney rank sum test). In contrast, pAra h 4 injected mice showed consistently higher IgG1 responses than mice injected with pAra h 1 (pAra h 1 mice), and the IgG2a signals were usually less than the IgG1 signal, though the latter difference did not reach statistical significance. The same profile types were obtained 4 weeks after the first injection but with a lower

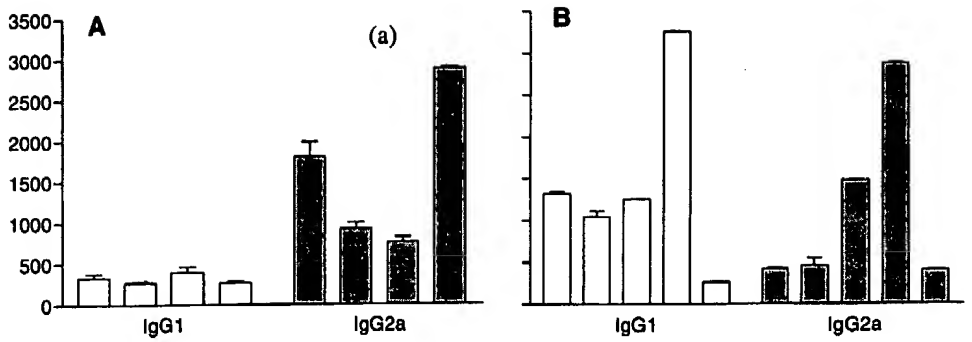

Figure 2. IgG1 and IgG2a reponse after 100 μg ID administration of pAra h 1 or pAra h 4 in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 100 μg in PBS of pAra h 1 (A) and pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 15 000[e]. The enzyme activity was stopped after 20 min. (a) in the pAra h 1 immunized mice, the IgG2a responses were consistently higher than the IgG1 responses ($P < 0.026$, Mann–Whitney rank sum test).

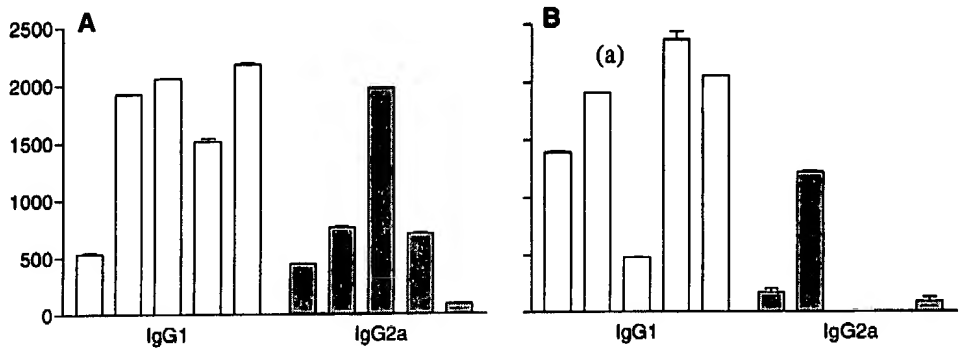

Figure 3. IgG1 and IgG2a response after ID administration of 100 μg of pAra h 1 or pAra h 4 in BALB/c mice. Groups of BALB/c mice were injected ID in the tail with 100 μg in PBS of pAra h 1 (A) or pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 15 000[e]. The enzyme activity was stopped after 20 min. (a) the IgG1 response to pAra h 4 was consistently higher than the IgG2a response in these mice ($0.032 < P < 0.056$, Mann–Whitney rank sum test).

intensity, and the same pattern for Ara h 1 immunized mice was also obtained with plates coated with purified Ara h 1 protein (data not shown). IgE was detected in pAra h 4 mice with high IgG1 levels, e.g., mouse number 4 (Fig. 2), but not in pAra h 1 mice.

ID Injection of 100 μg of pAra h1 or pAra h 4 in BALB/c mice

The protocol of immunization and bleeding for groups of BALB/c mice was the same as that above. Like the SKH-Hr1 mice, both groups had IgG1 and IgG2a responses. The IgG1 response was equivalent between the pAra h 1 ($n = 5$) and pArah4 groups ($n = 5$), but the IgG2a response was lower in the pAra h 4 group (Fig. 3). The IgG1 response to pAra h 4 was consistently higher than the IgG2a response in these mice ($0.032 < P < 0.056$). IgG2a was detectable in the number 3 and 4 mice of pAra h 4 group at a lower dilution (1/5000). As with the SKH-Hr1 mice, the ratio of IgG2a/IgG1 was higher in the pArah1 group than in the pArah4 group. Despite the high level of IgG1, no IgE could be detected in either group.

ID injection of 1 μg of pAra h 1 or pAra h 4 in SKH-Hr1 mice

Two groups of SKH-Hr1 mice were injected with 1 μg of plasmid pAra h 1 ($n = 3$) or pAra h 4 ($n = 4$). Immune response could not be detected at a higher dilution than 1/500. The profiles for both groups were similar to the profile obtained with 100 μg. Nevertheless the ratio IgG2a/IgG1 was lower with 1 μg of pAra h 1 than with 100 μg. No IgE could be detected in either group (Fig. 4).

ID injection of 1 μg of pAra h 1 or pAra h 4 plus 99 μg of blank plasmid in SKH-Hr1 mice

Two groups of SKH-Hr1 mice were injected with 1 μg of plasmid pAra h 1 ($n = 4$) or pAra h 4 ($n = 5$) plus

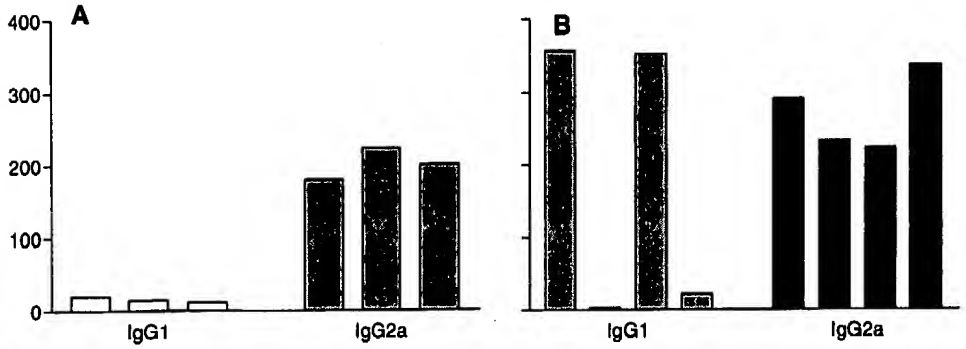

Figure 4. IgG1 and IgG2a response after ID administration of 1 mg of pAra h 1 or pAra h 4 in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 1 μg in PBS of pAra h 1 (A) and pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 500^e. The enzyme activity was stopped after 20 min.

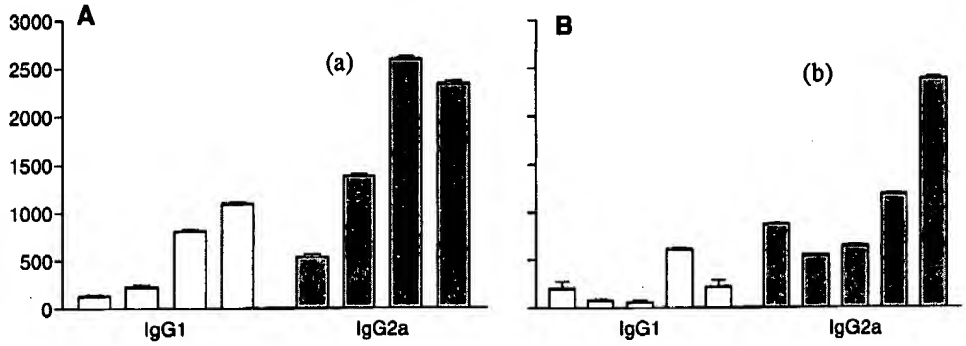

Figure 5. IgG1 and IgG2a response after ID administration of 1 μg of pAra h 1 or pAra h 4 + 99 μg of blank plasmid in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 1 μg in PBS of pAra h 1 (A) and pAra h 4 (B) + 99 μg of blank plasmid. A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 5000^e. The enzyme activity was stopped after 20 min. (a) the relative responses of IgG1 and IgG2a for pAra h 1 showed a statistically significant predominance of IgG2a ($P < 0.05$, Mann–Whitney rank sum test). (b) the relative responses of IgG1 and IgG2a for pAra h 4 showed a statistically significant predominance of IgG2a ($P < 0.01$, Mann–Whitney rank sum test).

99 μg of a blank plasmid in order to increase the ratio of CpG to recombinant protein expressed. pcDNA3 and blank plasmid (pCMVi) contain respectively 26 and 23 CpG. For both groups the magnitude of the immune response was lower than with 100 μg but higher than with 1 μg alone, showing the adjuvant effect of DNA (Fig. 5). The profile obtained in the pAra h 1 group was similar to that obtained after the 100 μg injection. The individual mice in both groups showed similar profiles. The IgG2a/IgG1 ratio was very close for both groups. The relative responses of IgG1 and IgG2a for both pAra h 1 and pAra h 4 showed a statistically significant predominance of IgG2a ($P < 0.05$ and $P < 0.01$, respectively). No IgE could be detected in either group.

Discussion

In this paper, we investigated various issues linked to genetic immunization with plasmid DNA encoding allergens. In our results, we showed that administration of pAra h 4 elicited a mixed Th1–Th2 immune response with detectable IgE in some mice or at least a Th1 response with a large amount of IgG1. In contrast, administration of pAra h 1 elicited a more classical Th1 response for genetic immunization with low amounts of IgG1. Previous studies have found that IgE can be detected after genetic immunization, as well. In our laboratory, we previously showed that administration of a plasmid encoding bovine beta-lactoglobulin (BLG) could elicit BLG specific IgE in some mice (14) that was correlated

with the quantity of IgG1. In their experiments, Raz et al. also detected very small amounts of IgE (4).

Immune reponses to proteins are known to be highly dependent on the nature of the antigen. In 1998, Lee et al. showed that for genetic immunization, using plasmids encoding the hemagglutinin and the nucleoprotein of influenza virus, the magnitude of the immune response and the degree of Th1 bias from CpG sequences depended on the antigen nature (15). In contrast to our results, the ratio IgG2a/IgG1 always favored IgG2a. Isoforms of major allergens of birch pollen, Bet v 1a and Bet v1d, on the other hand, induced different immune responses after genetic immunization (16). The humoral reponse was similar, but the cytokine profile and proliferation test showed different results. In contrast to our results, they could not detect any IgE, despite a high IgG1 level. The effect of CpG motifs *in vivo* depends also on a variety of parameters like the nature of the antigen and the immunization modality (17).

Immunization with plasmid DNA does not always induce a Th1 dominated response. The nature of plasmid DNA induced response is also dependent on the method of inoculation (18). DNA delivered by a gene gun produced a Th2-like response, while immunization by ID or IM with the same plasmid produced a Th1-like response.

Immune responses after genetic immunization are also influenced by the cellular location of expressed antigen (19). Plasmid DNA encoding secreted ovalbumin generated 50- and 100-fold higher levels of IgG1 than the cytoplasmic or membrane bound ovalbumin form. These results were confirmed by administration of a plasmid encoding for BLG, which elicited a large amount of specific IgG1 (14). BLG is naturally secreted in cow's milk and was found to be partially secreted by transfected cells and could even be detected in the sera after genetic immunization (20). The proteins belonging to the 11S globulin family, like Ara h 3 or Ara h 4, are storage proteins and so are unlikely to be secreted.

The immune response also depends on the mouse strain. Administration of plasmid encoding Ara h 2 in C3H mice induced an IgG2a and IgG1 response while in BALB/c or AKR mice it stimulated only an IgG2a response (21). In contrast, in our experiments we have a much higher IgG1 response in BALB/c than in SKH-Hr1 for each allergen. BALB/c has been shown to be Th2-biased for many other antigens (22).

The high IgG1 response to pAra h 4 is significant because in some cases it has been reported that anaphylactic reactions can be mediated by an isotype other than IgE (23). Indeed, IgE knock-out mice produced IgG2a, IgG1 and IgM after ovalbumin (OVA) sensitization but no IgE, and yet exhibited an anaphylactic reaction after OVA challenge. Oshiba et al. demonstrated that immediate hypersensitivity could be passively transferred not only by allergen-specific IgE, but also by IgG1, and it was not transferred by IgG2a or IgG3 (24). These studies demonstrate that IgG1 antibodies can play a role as important as IgE in the induction of immediate hypersensitivity in mice.

The immune response to allergens after genetic immunization is the result of two contradictory influences. The nature of the allergen itself promotes a Th2 response, while the presence of ISS sequences in the plasmid strongly stimulate cytokines promoting a Th1 response. For most antigens, the Th1 bias is predominant using genetic immunization, but with allergens, the relative strength of the Th2 bias of the antigen and the Th1 bias of the ISS sequences results in a more variable, mixed response. As a result, it is not surprising that we can modulate the immune response by changing the ratio ISS to the recombinant protein expressed.

Ara h 3 and Ara h 4 were cloned independently by two different groups, Rabjohn et al. (13) and Kleber-Janke et al. (12), respectively. Both proteins belong to the 11S protein storage family, which is a multigene family (25). The Ara h 3 clone was incomplete and so we used primers from the Ara h 4 sequence, because our goal was to obtain a complete cDNA for genetic immunization. From our source material, none of our cDNA clones had 100% homology to published Ara h 3 or Ara h 4 isoform sequences. However, all our clones contain a consensus cleavage site, Arg-Gly, and two cysteine residues implicated in a conserved disulfide bridge.

Further experiments will be required to determine if there is a difference in the protective effect of preventive genetic immunization with pAra h 1 and pAra h 4. It would also be interesting to better understand the properties of the expressed allergen of pAra h 4 that cause it to elicit a strong Th2 biased response.

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Molecular mechanisms in allergy and clinical immunology
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Immunostimulatory DNA and applications to allergic disease

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The vertebrate immune system reacts to certain sequences of DNA with a strong T_H1-inducing innate response. These sequences, termed immunostimulatory DNA sequences, are not fully defined but generally consist of a central nonmethylated CG dinucleotide, flanked by less highly conserved sequences (hence the alternate name CpG motifs). These sequences seem to be rare in vertebrates but relatively common in many lower organisms, including bacteria and viruses. It is likely that these sequences represent a danger signal to the immune system; a powerful T_H1 response is induced against colocalized foreign antigen. This can be used to modify an allergic response away from a pathogenic T_H2-dominated immune profile toward a nonpathogenic and even protective T_H1 profile. (J Allergy Clin Immunol 1999;104:902-10.)

Key words: Immunostimulatory DNA sequences, CpG motifs, DNA immunotherapy, DNA immunotherapeutics, allergic disease

Abbreviations used

APC:	Antigen-presenting cell
BCG:	Bacillus Calmette Guerin mycobacterium
CTL:	Cytotoxic T lymphocyte
dsRNA:	Double-stranded RNA
ISS:	Immunostimulatory DNA sequences
JNK:	c-Jun NH2-terminal kinase
MAPK:	Mitogen-activated protein kinase
NF-κB:	Nuclear factor-κB
NK:	Natural killer
ODN:	Oligodeoxynucleotide
ps:	Phosphorothioate
ROS:	Reactive oxygen species

It has recently been appreciated that DNA carries information about its originating organism in addition to its genetic blueprints. Rodent and primate immune systems react strongly to certain sequences (immunostimulatory DNA sequences [ISS]) that are very rare in vertebrates but are present in many lower, potentially pathogenic organisms. DNA containing these sequences is apparently perceived as likely to be associated with a dangerous invader and is thus a powerful adjuvant for activation of the innate arm of the immune response, detailed below. Briefly, professional antigen-presenting cells (APCs) such as macrophages and dendritic cells produce IL-12, IL-18, TNF-α, and IFN-α/β, while altering their surface molecule profile, and, in the case of macrophages, becoming cytotoxic. B cells also alter their surface markers for enhanced antigen presentation while proliferating and producing IL-6 and IL-12. Natural killer (NK) cells become cytotoxic and produce early IFN-γ. This concerted response then biases the adaptive arm of the immune system toward a T_H1 response against any locally encountered foreign proteins. Many researchers are attempting to harness the potency of T_H1 immune activation, the favorable safety profile, and the ease of use of ISS-containing DNA (oligodeoxynucleotides [ODNs] and plasmid) to treat or prevent disease.

IMMUNOSTIMULATORY NUCLEOTIDES: HISTORICAL PERSPECTIVES

Freund's famous adjuvant consisting of mycobacterial extract in oil immersion was initially characterized 60 years ago[1] yet remains one of the most potent modalities for generation of a T_H1 response. CFA contains many immunologically active substances, but it is likely that some of its activity is the result of mycobacterial genomic DNA.[2]

In the 1960s it was shown that natural double-stranded RNA (dsRNA) and its more stable analog polyinosinic, poly-cytidilic ribonucleic acid could stimulate the antiviral response by production of IFN-α and IFN-β.[3,4] The IFN-induced, dsRNA-activated protein kinase is responsible for this phenomenon, which probably evolved as a mechanism for sensing the dsRNA produced during the life cycle of some viruses.[5,6]

Yet it wasn't until the 1980s that the first reports of the immunostimulatory properties of DNA appeared. Tokunaga and his group in Japan found that it was the mycobacterial genomic DNA activation of NK cells that

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TABLE I. Potent immunostimulatory DNA sequences

Sequence	Notes	References
Stimulatory activity shown reproducibly by several groups:		
AACGTT	One of the first sequences discovered; we and others use this sequence frequently; stimulates human cells	13-18
AACGTTCG	More active than AACGTT in our hands; this is sequence we use most often	2, 15
AGCGCT	One of first sequences discovered	13-15, 19
GACGTC	One of first sequences discovered; active as a 6-mer ODN; stimulates human cells	13, 15, 16, 20
GACGTT	Active as 6-mer ODN; stimulates human cells; modified flanking sequences can give good response with low TNF-α; this sequence has been used in many studies	20-23
Limited reports of stimulatory activity:		
AACGAT	Moderate B-cell activation	19
AACGCT		18, 19
ACGCGT	Active as a 6-mer ODN	20
AGCGTT		24
ATCGAT	Active as a 6-mer ODN	14, 20
CACGTG	Active as a 6-mer ODN	17, 20
CACGTT		25
CTCGAC		19
CTCGCA	From acute retrovirus genome	26
CTCGTA	From acute retrovirus genome	26
GACGAT		25
GACGCT		25
GACGTA		25
GACGTG		25
GGCGTT		25
GTCGAC	Active as a 6-mer ODN	20
GTCGAT		17, 27
GTCGCT	May stimulate human cells	21, 24, 27
GTCGTC	May stimulate human cells	21
GTCGTT		17, 24
TACGTA	Active as a 6-mer ODN	20
TACGTT		25
TCGCGA	Active as a 6-mer ODN	14, 20
TGACGTT	Version of GACGTT that is claimed to be optimal mouse B-cell stimulatory sequence	24
Conflicting reports of stimulatory ability:		
CGCGCG	Has been shown to be stimulatory,[14] has been shown to be inhibitory[28]	14, 28
GACGGT	Demonstrated activity,[16,24] demonstrated lack of activity[25]	16, 24, 25
GCGCGC	Has been shown to be stimulatory,[14] has been shown to be inhibitory[28]	14, 28
GGCGCC	Report likely activity,[29] but not as 6-mer ODNs[20]	20, 29
GGCGGT	Demonstrated moderate activity,[24] demonstrated lack of activity[25]	24, 25
GTCGGT	Demonstrated low stimulatory ability,[17] demonstrated high stimulatory ability[19,24]	17, 19, 24
TGCGCA	Report likely activity[29] but not as 6-mer ODNs[20]	20, 29

This table is a compilation of the central core sequences of ODNs that have been shown to be immunostimulatory. However, except where noted that the 6-mer ODN is stimulatory, the actual ODN used was longer than this core sequence. There is some indirect evidence, but no direct evidence, that these core sequences constitute the fundamental stimulatory units (see text for details). It is also important to note that most of these studies referenced used different assays for immune stimulation under different treatment conditions. The most common assays include induction of proliferation (B cells), cytokines (APCs and NK cells), or cytolytic activity (mostly NK cells, some macrophages) on incubation of murine splenoctyes with short (12-30-mer) phosphorothioate (ps)-modified ODNs. The work to define the exact motifs required for immune stimulation is still in progress; this table is not meant to be a final and authoritative listing, only a general guide to what has been shown to date. There are other sequences that have been demonstrated to have lesser but significant immunostimulatory ability; only the most potent sequences are shown.

was responsible for the potent antitumor activity of an extract of *Mycobacterium bovis* Bacillus Calmette-Guerin mycobacterium (BCG).[7-12]

DEFINING IMMUNOSTIMULATORY SEQUENCES

Even after intensive attempts to precisely define the DNA sequence structure required for immune stimulation, this most fundamental aspect of ISS is only partially understood (Table I). After their initial characterization of the immune stimulatory properties of mycobacterial DNA, Tokunaga and colleagues found that 6 of 13 synthetic 45-mer ODNs taken from the known sequence of the mycobacterial genome had some activity. Further dissection with overlapping and shorter ODNs led these researchers to the conclusion that a few palindromic hexamers containing CpG dinucleotides were the active units.[13,14,20,29,30]

Later studies by Krieg et al[19] revealed a tentative con-

sensus sequence of single-stranded 5′-purine-purine-C-G-pyrimidine-pyrimidine-3′ to have optimal B cell stimulatory ability. This consensus also seems to hold for production of IL-6, IL-12, and IFN-γ by mouse splenocytes[18] and for NK activation in mouse and human cells.[17] However, this consensus is only a loose rule: there are many sequences that are stimulatory that do not follow the motif and many sequences that would be included within the consensus that are not particularly stimulatory. One aspect of ISS that does appear to be generally required is the inclusion of CpG, preferably near the center of an ODN.[14,19] Nevertheless, some sequences without CpG have some stimulatory ability,[21] which is why we prefer the functionally defined name ISS to the structurally defined name CpG motif.

There is a further layer of complexity in the structural requirements for ISS activity—epigenetics. It has been shown that ISS lose their stimulatory ability with C-5 methylation of the cytosine at the CpG core.[17,19,31,32] This is particularly interesting because vertebrates methylate about 70% of their CpG DNA (which is already reduced about 5-fold in frequency) and nearly all of their CpG sequences that are not in active promoter or enhancer regions.[33,34] Methylation within promoters and enhancers shuts down transcriptional activity and is used in long-term gene regulation, X-chromosome inactivation, and suppression of invading and endogenous viruses and transposons.[35,36] Additionally, methylation is believed to be responsible for the drastic reduction in frequency of CpG in the vertebrate genome. ^{me}CpG is a hotspot for mutation to TpG during replication, and in fact organisms with low CpG tend to have more TpG and its complement CpA than expected.[33] Thus it makes sense that there are clusters of unmethylated CpGs (CpG islands) found near the promoters of genes (where CpGs are not methylated) because the rest of the genome has undergone methylation and mutation over evolutionary time. This is in contrast to lower organisms, where the frequency of CpG dinucleotides varies widely but in general is near the expected occurrence of 1:16, and the C-5 positions on the cytosines are not as heavily methylated.[37] Not all CpGs are ISS, and we have found that the frequencies of active ISS are even further reduced in mammalian genomes and are altered in pathogen genomes (Van Uden and Raz, manuscript in preparation) beyond dinucleotide CpG frequencies.

Although ISS are generally considered by researchers in this field to be modular 6-mer units, it has been difficult to determine the minimum stimulatory motif length. One study showed that a minimum length of 18 bases was required but that a length of 22 bases gave greater activity.[38] Another study demonstrated good activity with a 15-mer ODN.[17] Still another study used cationic lipid transfection to show a stimulatory effect with a 6-mer ODN.[20] Phosphorothioate (ps) ODNs are nuclease resistant and were shown to have activity when as short as 8 bases.[19] It is possible that the minimum motif is around 6 bases but that exonucleases within target cells destroy short ODNs before they can have significant activity. Another possibility consistent with some of our new work is that the CpG core is the primary active unit and that a variety of flanking sequences are permissive for activity. This would cause each ISS to appear to have a different minimum length because crucial flanking bases would be variably distant from the core (Van Uden and Raz, manuscript in preparation).

Nuclease resistant ps ODNs can be as much as 200-fold more potent than natural phosphodiester ODNs while apparently maintaining the original sequence specificity and spectrum of stimulation.[19] The ps modification replaces 1 of the 2 nonbridging oxygens of the internucleotide phosphate with a sulfur and thus prevents nuclease activity and greatly extends the half-life.[39,40] The ps ODNs have been used extensively for antisense, are commercially available, have been approved for use in humans, and are therefore gaining in popularity.

Almost all this work has been done in mice, but it is clear that human cells and primates also respond to ISS, sometimes with different sequence specificities. More work in this area is required and is underway, but one study has addressed some differences between sequences that stimulate mouse splenocytes and those that stimulate human peripheral B cells.[21]

Recently an entirely new functionality of ISS has been discovered. Repeating sequences containing Gs and Cs have been found that specifically neutralize ISS activity, which have been named CpG-N motifs.[28] We have found another class of sequences that not only inhibit the T_H1-biasing activity of ISS but also promote a T_H2 response (Van Uden and Raz, submitted for publication). Therefore there seems to be an important balance between the number and types of sequences in any given DNA that determines its ability to stimulate the immune system. This concept is likely to be important in understanding the net stimulatory properties of plasmids and genomes.

DNA IMMUNOSTIMULATION: NK CELL ACTIVATION

Activation of NK tumoricidal activity was the first effect of ISS that was described,[9] and it has subsequently been shown that ISS-activated NK cells are more than 90% of the early IFN-γ–producing cells (later $CD4^+$ cells contribute the majority of IFN-γ).[41] Purified NK cells are not directly activated by ISS; the IL-12, TNF-α, and IFN-α/β produced by APCs are required (however, lack of B and T cells in mice with severe combined immunodeficiency syndrome does not reduce NK activation) (Fig 1).[13,17,42] Although most of the NK characterization has been done with mouse cells, human blood NK cells are also responsive to ISS.[17]

DNA IMMUNOSTIMULATION: MACROPHAGE AND DENDRITIC CELL ACTIVATION

ISS directly activate macrophages and macrophage-like cell lines[17,31,42,43] but indirect activation by IFN-γ

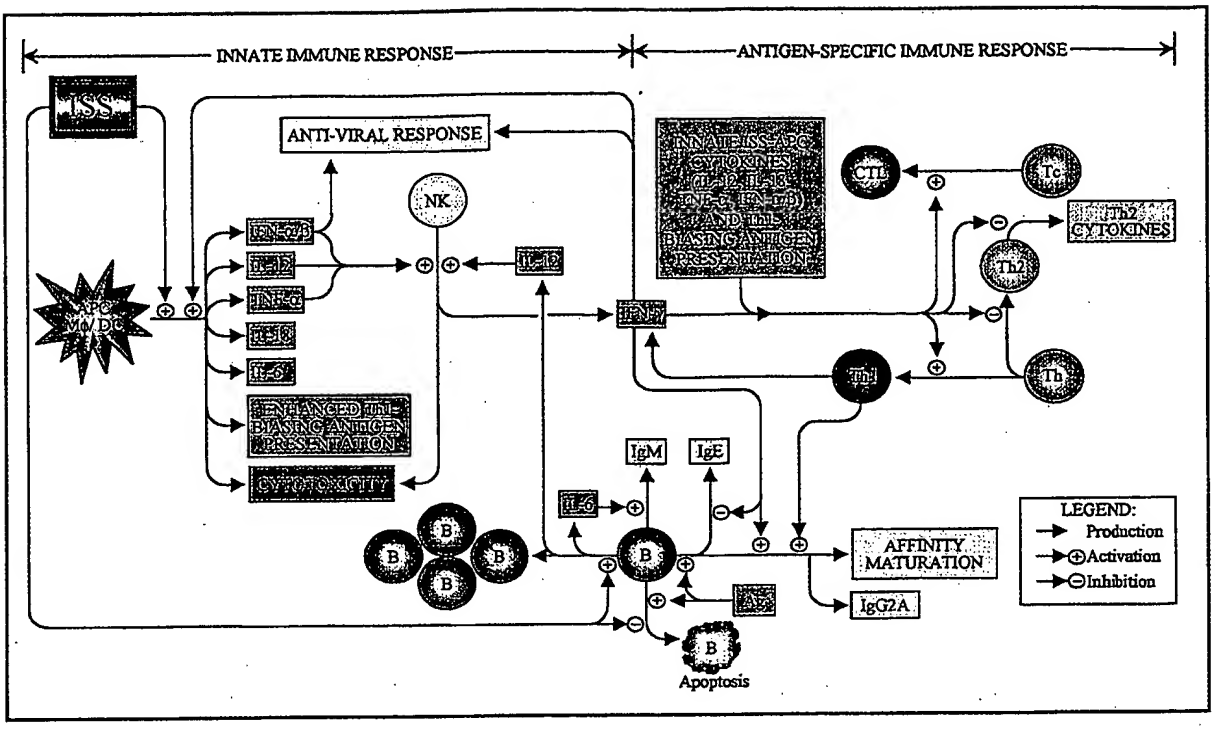

FIG 1. Intercellular mechanisms of ISS activation. This diagram summarizes known intercellular mechanisms of ISS activation of innate and antigen-specific arms of immune response, as detailed in text. Note that direct effects of ISS are apparently limited to stimulation of professional APCs and B cells within the innate arm and that it is the products of these stimulations that then drive the characteristic T_H1 development in the antigen-specific arm. *Mϕ*, monocyte/macrophage; *DC*, dendritic cell; *Ag*, antigen; *Tc*, naive CD8+ CTL precursor; *Th*, naïve CD4+ T_H1 or T_H2 precursor.

appears to account for the majority of quantitative activation of macrophages in mixed cell cultures and in vivo.[11,13] ISS-activated macrophages become cytotoxic and produce the T_H1-biasing cytokines TNF-α, IL-12, IL-18, and IFN-α/β.[2,15,31,42,43] These cytokines stimulate IFN-γ production by NK cells in the innate phase of the response to ISS and by T_H1 cells in the adaptive phase. IFN-γ, in turn, potently activates macrophages further, forming a positive feedback loop (Fig 1). The reactivity of macrophages was defined in mouse systems, but human macrophages are similarly activated by ISS.[15] More recently it has been appreciated that the antigen processing and presentation apparatus of macrophages is also amplified on ISS stimulation. Bone marrow–derived macrophages treated with ISS-ODN up-regulate their expression of class I MHC, CD40, intercellular adhesion molecule-1, and CD16/32 (IgG receptor for bound antigen internalization and antibody-dependent cellular cytotoxicity) and consequently function more effectively as APCs.[44]

Dendritic cells are similarly activated by ISS. They produce IL-12, IL-6, and TNF-α in response to ISS, and they increase their class II MHC and B7-2 levels.[45,46] ISS-treated dendritic cells also function better as APCs in allogeneic mixed lymphocyte reactions and with superantigen stimulation[45] and may be particularly important in ISS-stimulated responses in vivo because of their proposed role in priming of immune responses.[47] Thus the 2 most important classes of professional APCs are potently stimulated by ISS.

DNA IMMUNOSTIMULATION: B-CELL ACTIVATION

B cells also are directly stimulated by ISS. The profound mitogenic effect of ISS on B cells was first described in 1991[48] and more than 95% of purified primary B cells in vitro will enter the cell cycle.[19] In addition, purified B cells stimulated with ISS will produce polyclonal IgM antibodies independent of T-cell help or antigen exposure.[19,21,49] ISS also rescues B cells from surface IgM cross-linking–induced apoptosis.[25,27,50,51] B cells produce high levels of IL-6 and IL-12 in direct response to ISS (Fig 1).[18,24,49] B-cell proliferation is independent of IL-6 production (as determined with blocking antibodies and with knockout mice) (Martin-Orozco and Raz, unpublished observation), but neutralization of IL-6 reduces IgM production by 90%,[18,24] suggesting autocrine stimulation (Fig 1). The relative importance of B cell versus macrophage and dendritic cell production of IL-12 in establishment of the T_H1 response is unclear.[15,18,42]

The surface molecule expression profile of B cells is also drastically altered by ISS activation. Class I MHC,

class II MHC, B7-1, B7-2, CD40, CD16/32, intercellular adhesion molecule-1, IFN-γR, and IL-2R are all up-regulated in vivo or in vitro, whereas CD23 (the low-affinity IgE receptor) is down-regulated.[44]

Although ISS can effectively activate B cells as part of the innate response (in an antigen-independent fashion), ISS also acts as a powerful costimulatory signal for B cells, which receive signals through their B-cell reactivity. Proliferation, IL-6 production, and IgM secretion are all synergistically increased with low-level surface B-cell reactivity cross-linking combined with ISS-ODN treatment (Fig 1).[19,24] In addition, ISS appears to allow cooperation between the cellular and humoral responses. Normally IFN-γ inhibits the IgM produced by LPS activation of B cells, but with ISS IFN-γ increases the IgM and IL-6 produced.[49] Human B cells also respond to ISS; 95% of human peripheral B cells stimulated with ISS-ODN increase their expression of B7-2 and IL-2R.[21]

DNA IMMUNOSTIMULATION: T-CELL ACTIVATION AND T_H1 BIASING

T cells are also activated by ISS, although not in a direct way. Simple stimulation of purified T cells with ISS-ODN does not result in activation. Treatment of splenocytes with α-CD3–activating antibody combined with ISS-ODN leads to slightly increased levels of CD69 and B7-2 on T cells, but this effect is mediated by IFN-α/β.[52] Similar treatments with ISS-ODN and α-CD3 led to increased levels of IFN-γ and to reduced levels of the T_H2 cytokines IL-3, IL-4, IL-5, and GM-CSF[53](Van Uden and Raz, submitted for publication). Blocking studies have revealed that IFN-α/β, IL-12, and IFN-γ from APCs and NK cells contribute to the above alteration in the cytokine profile of T cells.[53]

T cells are not required for the innate response to ISS,[17,21,31,42,43,48] but both $CD4^+$ and $CD8^+$ T cells are vital for the characteristic T_H1 response to genetic vaccination.[54,55] The T_H1 response to genetic vaccination and to ISS-ODN/antigen codelivery includes generation of $CD8^+$ cytotoxic T lymphocyte (CTL), T_H1 cells, IgG2a > IgG1, and IFN-γ > IL-4, IL-5, and IL-10 (Fig 1).[2,15,23,54,56-60] Generation of this strong T_H1 response can prevent the generation of, or even suppress a continuing, T_H2 response to the same antigen (see below). We have shown that this crucial profile of adaptive immune response activation in gene vaccination is at least largely the result of the presence of ISS in the backbone of all plasmids, which are bacterially derived.[15] Therefore the plasmid can be thought of as 2 distinct immunologic components: the antigen expression cassette and the various ISS located throughout that act as T_H1-promoting adjuvants.[61]

One prominent exception to the T_H1 rule is the T_H0/T_H2 bias usually seen with intradermal particle–mediated transfer of plasmid DNA (the gene gun).[62,63] This system is significantly different in that only about 1% of the amount of adjuvant DNA is administered relative to syringe injection and because a significant quantity of potentially immunologically active gold particles are also present.[2,64]

Recent reports of the first generation of gene vaccination trials in monkeys and humans have met with only partial success.[65-68] One of the major limitations to successful gene vaccination is the very low level of antigen expression, but we and others have reported that coadministration of ISS-ODN with protein results in strong CTL, antibody, and T_H1 cytokine production.[2,23,69] An even more potent way to increase the response to the antigen is to chemically conjugate it to the ISS-ODN adjuvant to facilitate entry into the same APCs (Raz, manuscript in preparation).

MOLECULAR MECHANISMS

The molecular pathway from sensation of ISS-containing DNA to production of the characteristic cytokines and cell surface molecules is still largely undiscovered territory and an area of active investigation. Probably the most important of these areas is identification of the mechanism directly responsible for sensing ISS in or on a cell. There is likely a sequence-specific DNA binding protein(s) that is an ISS receptor, but there are still no published reports of such a protein. There is some evidence that ISS need to be internalized for activity. ISS are generally more potent and can be shorter when they are directly transfected into cells.[20,70] Also, ISS-ODNs covalently bound to a solid support do not stimulate mouse B cells.[19]

The intracellular signaling events after ISS activation have been partially investigated. Several common pathways have been shown to not be involved: tyrosine phosphorylation, inositol triphosphate generation, calcium ion (Ca^{++}) flux, protein kinase C activation, protein kinase A activation, and nitric oxide production.[19,24] The first positive finding was that reactive oxygen species (ROS) are generated within 20 minutes of stimulation with ISS, before IL-6 messenger RNA, which appears at 30 minutes.[24] Blockade of ROS activation with antioxidants completely blocks B-cell activation by ISS.[24] Nuclear factor-κB (NF-κB) has also been shown to be activated, and it is apparently necessary for ISS stimulation, although the NF-κB inhibitors used are not entirely specific.[31,50] The time course of NF-κB activation is similar to that of cytokine messenger RNA induction, which is inconsistent with its proposed central role in ISS signal transduction. ROS activate NF-κB, and both are involved in a great variety of immunologic signaling cascades and are thus not specific to ISS.

The mitogen-activated protein kinase (MAPK) and stress kinase pathways are also activated by ISS. Specifically, c-Jun NH2-terminal kinase (JNK), JNK kinase 1, p38, activating transcription factor-2 (ATF-2), c-Jun, MAPK-activated protein kinase-2, and activator protein-1 (AP-1) have been implicated in ISS signaling.[71,72] The p38 pathway appears to be crucial because its blockade results in lack of cytokine production and of downstream signaling component activation.[71,72]

Curiously, blockade of endosome acidification/maturation by chloroquine, monensin, or bafilomycin A inhibits the earliest aspects of the ISS response, includ-

ing the known signaling pathways.[71,72] This could represent the ISS escaping the endosome, or perhaps some kind of potentiation or sensing of ISS.

A host of apoptosis-related genes are differentially regulated when ISS is used to block apoptosis in B cells. ISS appears to up-regulate myn, bcl-2, bcl-x_L, and egr-1, whereas c-*myc* was up-regulated in one study and down-regulated in another.[27,73]

There is not yet a clear picture of how these disparate signaling clues form the greater picture of ISS activation of immune genes, but it is apparent that there are multiple interacting pathways.

CLINICAL APPLICATION: ALLERGIC DISEASE

There are many different potential areas where the ISS T_H1-inducing profile of adjuvanticity would be useful, such as in infectious disease vaccines, in cancer therapeutic vaccines, and in infectious disease immunotherapy. However, this review will focus on the implications for treatment of allergic disease.

Asthma and other allergic diseases are largely mediated by profound T_H2 responses against environmental antigens. It has been proposed that strong T_H1 responses can suppress and even down-regulate T_H2 reactions in an antigen-specific manner, thus ameliorating the disease.[74] The only currently available option for antigen-specific treatment is standard allergen immunotherapy, which involves injecting sensitized individuals with extremely small but increasing doses of allergen over the course of years, thus diverting the immune response. This immunotherapy only works for a select set of allergic diseases and carries a small but significant risk of side effects, including anaphylaxis.[75] We and others have shown that the T_H1-inducing properties of ISS can be used to prevent or treat allergic disease in rodent models.[15,53-56,76-79]

It has been shown that genetic vaccination encoding a model antigen, β-gal, leads to a T_H1 response. This response dominates over subsequent or previous T_H2 sensitizations with β-gal in alum. IgE in particular is almost completely inhibited when plasmid is given before β-gal/alum, and IgE is drastically reduced when plasmid is given after β-gal/alum.[56] In a prophylaxis model, another group has shown that vaccination with a plasmid encoding the dust mite allergen Der p 5 inhibits the induction of allergy at a later time as measured with IgE production, airway hyperreactivity, and histamine release. Interestingly, this protection could be adoptively transferred by $CD8^+$ T cells.[55,80] It has been shown that this T_H1-promoting and T_H2-inhibiting effect of genetic vaccination can be largely accounted for by the presence of ISS in the plasmid backbone of genetic vaccines.[15,32]

In addition to these antigen-specific effects of ISS as a T_H1-promoting (and therefore T_H2-inhibiting) adjuvant, ISS also have direct T_H2-inhibiting effects. We have demonstrated that only one injection (or mucosal administration) of ISS-ODN given before allergen challenge to allergically sensitized mice inhibits lung and blood eosinophilia better than does 7 daily injections of dexamethasone. Levels of antigen-specific IL-3 and IL-5 and airway hyperreactivity were also reduced, whereas IFN-γ levels were increased.[53] This phenomenon of immediate inhibition of the allergic response by ISS-ODN has recently been confirmed with a different allergen, ragweed. This investigation further showed that the longer-term T_H1 diversion of the allergen-specific response is stable for at least 6 weeks.[78] This observation has recently been extended to other allergic diseases. ISS-ODN administration has been shown to block the development of both the early and late phases of murine allergic conjunctivitis, even when it is given after allergen sensitization (Magone and Raz, submitted for publication). Although the above studies use ISS primarily as a pharmacologic inhibitor of established T_H2 immunity, the ability of ISS-ODN to act as a T_H2-inhibiting adjuvant in a mouse model of asthma with use of shistosome eggs has also been shown. When the T_H2-inducing eggs were given with ISS-ODN, lung eosinophilia, airway hyperreactivity, and lavage IL-4 were reduced and lavage IFN-γ and IL-12 were increased.[77]

Thus the use of ISS both as prophylaxis and treatment of allergic disease is promising. ISS have shown utility both as short-term antigen-nonspecific inhibitors of allergic inflammation and as adjuvants for long-term immune deviation. Studies in monkey models of allergic disease have also been very promising (Raz, unpublished data) and human clinical trials are not far away.

POTENTIAL SIDE EFFECTS

Advantages of ISS for use as adjuvants include their powerful activation of multiple immune cell types and of T_H1 responses. However, the immune system is delicately balanced between immunity and tolerance, between T_H1 and T_H2, and between inflammation and unresponsiveness. There is always the possibility of unwanted effects of the powerful immune stimulation that ISS delivers.

LPS is similar to ISS in that it is a conserved bacterial product that is recognized by the immune system as a danger signal and it stimulates the production of many of the same cytokines. A systemic overload of LPS, however, can lead to lethal septic shock by production of excessive IL-1 and TNF-α. Additionally, high levels of IL-6 are predictive of a poor outcome in septic shock.[81] ISS also induces the production of TNF-α and IL-6, so it is possible that it could lead to shock in environmentally or genetically sensitized individuals. It has been shown that mice given ISS followed by sublethal doses of LPS die of shock at an increased frequency (control at 0% to ISS-treated at 75%) in an IFN-γ–dependent manner.[41] In a separate study it was found that mice presensitized with D-galactosamine then given ISS also undergo lethal shock; this model demonstrated that TNF-α but not T, B, or NK cells are required.[43] Interestingly, these researchers found that some immunostimulatory sequences retain their ability to stimulate IL-12 production without

producing TNF-α and are safe in their shock model.[22] Although these reports demonstrate the possibility of shock in extreme cases of sensitization or concurrent LPS exposure, there has never been a reported case of ISS alone causing shock in any kind of healthy animal at any dose.

Another possibility is that ISS could cause excessive local inflammation, as seen with other powerful T_H1 adjuvants such as CFA. We and others have never observed gross inflammation in response to ISS in ODN or plasmid form in any experimental animals or humans.[57] There can be immune-mediated destruction of antigen-expressing tissue in gene vaccination, but this effect is antigen-specific, not a generalized inflammatory process.[82] In contrast, it has been reported that delivery of ISS-containing bacterial DNA or ODNs to the lung can cause increases in the levels of neutrophils and other immune cells while increasing lavage fluid levels of TNF-α, IL-6, and macrophage inflammatory protein-2.[83] Fluid from the lungs of patients with cystic fibrosis was able to reproduce this effect in mouse lungs, implying that the copious amounts of bacterial DNA found in these patients' lungs could be responsible for some of the immune-mediated damage seen clinically.[83] However, this study never shows any clinically significant inflammation in their mouse model, only correlates of inflammation that could also be interpreted as important for proper adjuvanticity. In addition, this same group also found that systemically administered ISS can actually reduce LPS-induced airway inflammation in an IL-12–dependent manner.[84]

When ISS-containing ODNs (ISS-ODNs) are injected into mice, the most obvious effect is a massive but transient splenomegaly within the first 2 weeks, primarily caused by extramedullary hematopoiesis in the spleen.[85] This is not likely to be relevant to humans because extramedullary hematopoiesis is not usually observed past the neonatal period and because this phenomenon has not been observed in humans and nonhuman primates treated with ISS-containing antisense ODNs.[86]

Many autoimmune diseases are T_H1 in nature, and the danger exists of precipitating disease in genetically or environmentally predisposed individuals. In one model of experimental allergic encephalomyelitis, T cells primed in vivo with myelin basic protein in CFA (which contains bacterial DNA), and then restimulated with antigen and ISS in vitro will exit quiescence and transfer disease to recipient mice in an IL-12–dependent fashion.[87] Another group of researchers has shown that bacterial DNA can generate anti-DNA antibodies in a model of systemic lupus erythematosus. To make the DNA antigenic, bacterial DNA is complexed with methylated bovine serum albumin in CFA and injected into mice. Normal healthy mice make anti-DNA antibodies and even develop low levels of proliferative glomerulonephritis and proteinuria.[88,89] When the mixture is given to preautoimmune NZB/NZW F1 mice, they develop antibodies that cross-react with mammalian DNA, but surprisingly they are actually protected from their spontaneous autoimmune disease.[90,91] There still are no examples of ISS directly causing any type of autoimmune disease in animal models.

SUMMARY AND PERSPECTIVES

Immunostimulatory DNA sequences in plasmid DNA or in ODNs potently and rapidly activate the innate immune system, triggering NK cells, macrophages, dendritic cells, and B cells. These cells produce antigen-nonspecific cytotoxicity, polyclonal IgM antibodies, and an environment that is both antiviral and conducive to generation of an antigen-specific T_H1 response. The resulting adaptive immune response consists of $CD4^+$ T_H1 cells, $CD8^+$ CTL cells, and high-affinity T_H1-type antibody (IgG2a in mice). This profile is especially well suited to attack intracellular pathogens and also to inhibit T_H2-mediated diseases such as allergy.

There are also cases in which the stimulatory properties of DNA should be minimized, such as in gene replacement therapy. The recent discovery of sequences that can inhibit the function of ISS, or even act as T_H2 adjuvants, opens up an exciting new arena for the modulation of the immunologic activity of DNA.

Most of the work on ISS has been in mice, but it is clear that human cells also respond. It may be important for those who wish to take ISS to the clinic to optimize sequences for potency and consistency in humans; it is already clear that the best sequences in mice are not necessarily the best in people.

ISS are also exciting from the basic science point of view. This system is assumed to have evolved to sense dangerous pathogens, but there are to date no reports of any experiments to determine the actual importance of ISS in host-pathogen interactions or what countermeasures successful pathogens may have evolved to avoid or take advantage of this system.

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CpG Oligonucleotides Are Potent Adjuvants for the Activation of Autoreactive Encephalitogenic T Cells In Vivo

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The mechanism of action of microbial adjuvants in promoting the differentiation of autoimmune effector cells remains to be elucidated. We demonstrate that CpG-containing oligodeoxynucleotides (ODN) can completely substitute for heat-killed mycobacteria in the priming of encephalitogenic myelin-reactive T cells in vivo. The adjuvanticity of the CpG ODN was secondary to their direct ability to induce IL-12 or to act synergistically with endogenous IL-12 to promote Th1 differentiation and encephalitogenicity. T cells primed in the absence of CpG with Ag and IFA alone appeared to be in a transitional state and had not undergone differentiation along a conventional Th pathway. Unlike Th2 cells, they expressed low levels of the IL-12Rβ2 subunit and retained the ability to differentiate into encephalitogenic effectors when reactivated in vitro under Th1-polarizing conditions. These results support the use of CpG ODN as adjuvants but also suggest that they could potentially trigger autoimmune disease in a susceptible individual. *The Journal of Immunology,* **2000, 164: 5683–5688.**

It has been appreciated for many years that autoimmune diseases, including multiple sclerosis (MS),[3] present as well as recur more frequently in the setting of infectious illness (1–9). Furthermore, to induce experimental autoimmune syndromes, it is often necessary to inoculate laboratory animals with the target autoantigen combined with microbial adjuvants. In the case of experimental allergic encephalomyelitis (EAE), an autoimmune demyelinating disease used as an animal model of MS, successful disease induction depends on the inclusion of heat-killed mycobacteria along with myelin proteins in the inoculum. In addition, many EAE protocols call for the coinjection of inactivated *Bordetella pertussis* (10). Administration of myelin Ags without microbial products (e.g., emulsified in IFA) fails to provoke disease and, in some instances, may actually result in a state of tolerance, whereby the recipient is resistant to subsequent attempts at disease induction (11, 12). It has been widely reported that lymph node (LN) cells and splenocytes from animals primed with Ags in IFA, as opposed to CFA, fail to mount proinflammatory responses. They do not secrete IFN-γ on challenge in vitro or mediate classic delayed type hypersensitivity reactions on challenge in vivo. Depending on the study, this has been attributed either to deletion/anergy of the Ag-specific T cells or to their differentiation along a Th2 pathway (13–16). On the other hand, under certain unusual circumstances, cells from animals primed repeatedly with neuroantigens in IFA have induced disease (17).

The mechanism of action of microbial adjuvants in promoting the differentiation of autoimmune effector T cells remains to be elucidated. The first aim of this study was to attempt to define the component(s) of whole microbial preparations that are responsible for their in vivo disease-promoting effects. We focused our efforts on the role of microbial DNA because it stimulates production of IL-12 (18–21), a cytokine that we and others have previously demonstrated to play a critical role in pathogenesis of EAE as well as other organ-specific autoimmune diseases (22–27). We demonstrate that CpG-containing oligodeoxynucleotides (ODN) that mediate some of the immunomodulatory functions of bacterial DNA could completely substitute for heat-killed mycobacteria and prime encephalitogenic myelin-reactive T cells in vivo. The second aim of the study was to further define the phenotype of autoantigen-specific T cells that are primed in the absence of microbial adjuvants. We demonstrate that T cells from animals primed with myelin basic protein (MBP) in IFA fully retain the capacity to differentiate into encephalitogenic T cells when reactivated under conducive (Th1-polarizing) conditions in vitro. Furthermore, our studies suggest that exposure to Ag in IFA partially activates T cells to differentiate along the Th1 pathway. Taken together, these observations have important implications both for our understanding of the role of environmental factors in the pathogenesis of autoimmune disease and for therapeutic strategies that use soluble Ags to tolerize or deviate the immune response.

Materials and Methods

Mice

Female SJL mice were obtained from the National Cancer Institute (Fredrick, MD) at 6–8 wk of age and housed in a pathogen-free facility. Mice were between 8 and 12 wk of age when experiments were initiated.

Peptides and oligonucleotides

MBP_{87-106} (VVHFFKNIVTPRTPPPSQGK) was synthesized and purified by HPLC by the Laboratory of Molecular Structure, Peptide Synthesis Laboratory (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). ODN were purchased from Operon Technologies (Alameda, CA) and were phosphorothioate modified to increase their resistance to nuclease degradation. The sequences were as follows: CpG ODN, ATAATCGACGTTCAAGCAAG; control (non-CpG) ODN, ATAATAGAGCTTCAAGCAAG (28). The LPS content of the

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; LN, lymph node; MBP, myelin basic protein; ODN, oligodeoxynucleotide; IL-12Rβ2, the β2 subunit of the IL-12 receptor.

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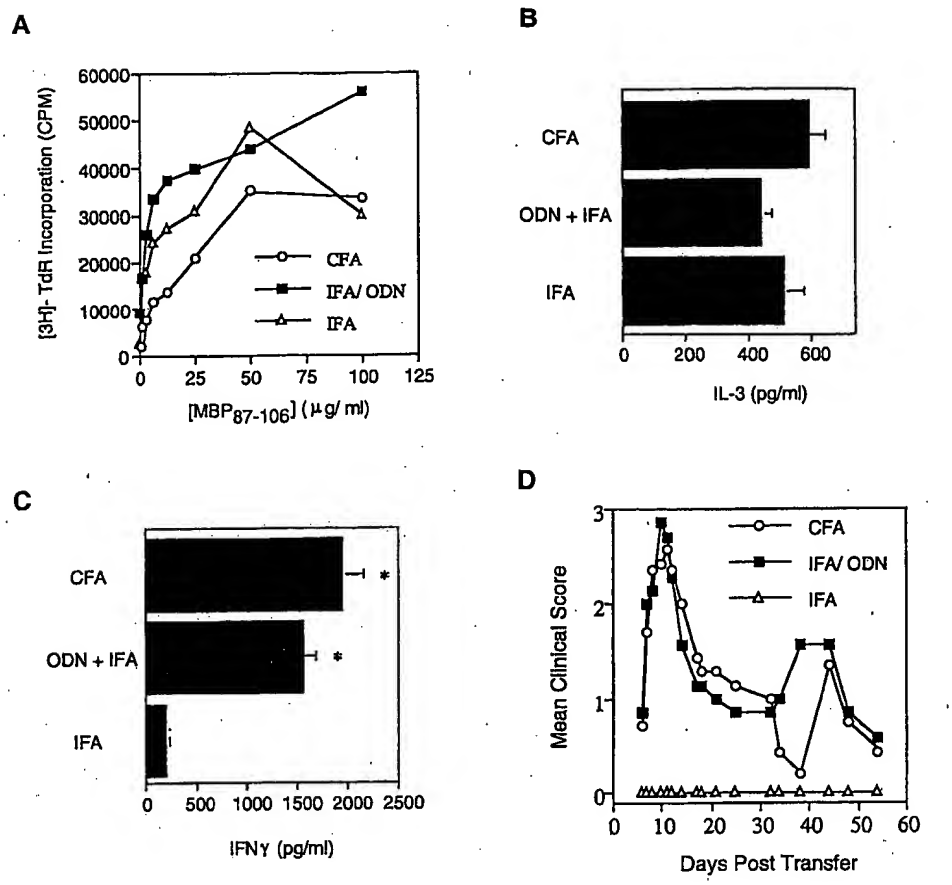

FIGURE 1. MBP-reactive T cells primed in the absence of microbial adjuvants expand but fail to differentiate into autoimmune effectors. CpG ODN are as effective as heat-killed mycobacteria in promoting encephalitogenicity. Female SJL mice were immunized with MBP_{87-106} in PBS and an equal volume of CFA, IFA plus CpG ODN, or IFA alone. On day 10, draining LNs were resected and made into single-cell suspensions. *A*, Proliferative responses of LN cells after 96 h of culture. The data are representative of five experiments with similar results. *B*, IL-3 production. *C*, IFN-γ production by LN cells cultured for 72 h with or without MBP_{87-106}. Cytokine production was generally below the limits of detection (12–24 pg/ml) in unstimulated cultures; measurable values were subtracted from the presented data. Mean values ± SD are shown for three experiments ($p < 0.005$). *D*, Ag-stimulated cells were harvested at 96 h, washed extensively, and injected i.p. into naive syngeneic recipients (5–8 mice/group). Recipient mice were monitored on a daily basis and rated on a 5-point scale as described in the text. In these experiments, all symptomatic mice progressed to a score of 2 or greater. Results are pooled from four experiments.

ODN was <1 ng LPS/mg DNA in all instances, as measured by *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD).

Generation of MBP_{87-106}-specific LN cells for cell transfer, mRNA measurements, and analysis of cytokine production

Mice were immunized with MBP_{87-106} (100 μg) emulsified in an equal volume of CFA, IFA, or IFA containing CpG ODN (60 μg) by s.c. injection at four sites over the flanks. In some experiments, mice were injected i.p. with a neutralizing mAb to IL-12, C17.8 (a gift of Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA), or normal rat IgG (Sigma, St. Louis, MO). Ten days later, draining LN (inguinal and axillary) were removed and processed as previously described (23). Cells were cultured with or without MBP_{87-106} (50 μg/ml) in RPMI containing 10% FCS and standard supplements. In some experiments, recombinant IL-12 (gift of S. Wolf, Genetics Institute, Cambridge, MA) or anti-IL-12 (C17.8) were added on day 1 of culture. Supernatants were sampled at 24-h intervals for quantification of cytokine levels. For Northern blot analyses, cells were harvested at 72 h, washed, and used for RNA extraction. For adoptive transfer, cells were harvested at 96 h and washed extensively. Viable cells were counted by trypan blue exclusion and injected i.p. into naive syngeneic recipients (5×10^7 cells/mouse). Recipients were examined daily by two observers, one of whom was blinded, for signs of EAE and rated for severity of neurological impairment as previously described (23).

Proliferation assays

LN cells (4×10^5 in 0.2 ml) were cultured with various concentrations of Ag or medium alone in triplicate for 4 days in 96-well round-bottom plates (Costar, Cambridge, MA) Wells were pulsed for the final 16 h of culture with 1 μCi [^{3}H]TdR (Amersham, Arlington, Heights, IL), and incorporated radioactivity was measured using a Betaplate scintillation counter (Wallac, Gaithersburg, MD).

Northern blot analysis

Total RNA was isolated from LN cell cultures using RNAzol RNA isolation solvent (Tel-Test, Friendswood, TX). Samples (10 μg total RNA per lane) were run on a 1.2% agarose gels containing MOPS buffer and formaldehyde and blotted onto a Hybond-N nylon membrane (Amersham). Membranes were baked for 2 h at 80°C and then probed for murine IL-12Rβ2 subunit or β-actin. Oligonucleotide probes were synthesized or purchased as previously described (29). PCR fragments (50 ng) were labeled with [^{32}P]dCTP using an oligolabeling kit (Pharmacia, Piscataway, NJ). Blots were prehybridized for 1 h at 42°C, followed by overnight hybridization with labeled probe at 42°C. Blots were then washed for 30 min in 2× SSC, 0.1% SDS buffer (room temperature) followed by 30 min in 0.1× SSC, 0.1% SDS buffer (55°C for IL-12Rβ2; 65°C for β-actin).

Cytokine ELISA

IL-2, IL-3, IL-4, IL-10, and IFN-γ were quantified using a sandwich ELISA technique based on noncompeting pairs of Abs as previously described (23). The lower limit of detection for each assay was 30 pg/ml or less.

Results

The adjuvant activity of CFA is duplicated by CpG ODN in IFA

Female SJL mice were immunized with MBP_{87-106}, the immunodominant peptide of MBP that binds I-A^s, emulsified with either IFA containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (CFA), IFA mixed with an immunostimulatory CpG-containing oligonucleotide (CpG ODN/IFA), or IFA alone. Draining LNs were removed 10 days later, dispersed into single-cell suspensions, and cultured with or without MBP_{87-106} to measure Ag-specific lymphoproliferation and cytokine production. LN cells from all three treatment groups mounted significant proliferative responses (Fig. 1*A*) and produced similar quantities of IL-3 (Fig. 1*B*). IL-2 was produced in low amounts by all groups (<100 pg/ml). Therefore, we concluded that MBP-reactive T cells were neither deleted nor anergized as a consequence of being primed in the absence of microbial adjuvants. Indeed, the fact that we were able to detect

equivalent MBP-specific responses during the secondary stimulation of polyclonal LN cell populations indicated that the autoreactive T cells had expanded in the IFA-primed mice as well as in their CFA- and CpG ODN/IFA-primed cohorts.

IL-4 and IL-10 were not produced in detectable quantities by LN cells from any of the groups. However, LN cells from donors immunized with Ag in CFA or Ag/CpG-ODN/IFA, but not Ag in IFA, produced IFN-γ and transferred disease after in vitro restimulation (Fig. 1, *C* and *D*). Thus, CpG-ODN reproduced the adjuvant effects of intact mycobacterial preparations by driving Th1 differentiation and by fostering the acquisition of disease-inducing properties by autoreactive T cells in vivo. There was no significant difference in the characteristics of MBP-reactive LN cells from donors immunized using IFA containing a control (non-CpG) ODN or IFA alone (our unpublished data). Therefore, the disease-promoting properties of CpG ODN appeared to be directly dependent on the presence of unmethylated CpG dinucleotides in its sequence.

The adjuvant effects of CpG-ODN are IL-12 dependent

We next used cytokine gene knockout mice to determine the relative importance of IFN-γ and IL-12 in the development of encephalitogenic T cells that were induced after priming with MBP/IFA/CpG ODN. We have previously demonstrated that IL-12-deficient (−/−) C57BL/6 mice are resistant to EAE induced by immunization with bovine MBP in CFA, whereas IFN-$\gamma^{-/-}$ mice are highly susceptible. Disease was suppressed in IFN-$\gamma^{-/-}$ mice by neutralization of IL-12 (24). These studies demonstrated that encephalitogenicity is induced by an IL-12-dependent, IFN-γ-independent pathway and that IFN-γ paradoxically suppresses EAE at some point in the pathogenic process.

In keeping with these earlier results, IL-$12^{-/-}$ C57BL/6 mice were resistant to EAE induction after immunization with MBP in CpG ODN/IFA. Furthermore, IFN-$\gamma^{-/-}$ mice were equally susceptible to EAE induced using either MBP in CFA or CpG ODN/IFA but did not develop disease when immunized with MBP in IFA alone (Fig. 2). These results suggest that the adjuvanticity of both intact mycobacteria and CpG-ODN is secondary to their ability to directly induce IL-12 or that they act synergistically with endogenous IL-12 to promote Th1 differentiation and encephalitogenicity. Furthermore, both microbial products achieve their adjuvant effects in the absence of IFN-γ.

We also examined whether the CpG ODN must be directly associated with MBP in the adjuvant emulsion for successful disease induction. IFN-$\gamma^{-/-}$ C57BL/6 mice were primed with bovine MBP in IFA s.c. and simultaneously injected with CpG ODN i.p., i.v., or in the foot pad. Whereas 100% of mice developed severe EAE when MBP_{87-106} and CpG ODN were combined in a single emulsion, they remained asymptomatic when MBP_{87-106}/IFA and CpG ODN were administered at independent sites (Fig. 2). This result suggests that CpG ODN cannot mediate their adjuvant effects at a distance and that the same APC must present MBP and produce CpG ODN-induced factors (such as IL-12), or neighboring APCs must perform these functions in tandem.

Ag in IFA partially primes Th1 effector cells

MBP-reactive LN cells from IFA-primed mice neither secreted detectable quantities of IFN-γ in vitro nor transferred disease into naive syngeneic recipients (Fig. 1, *C* and *D*). Because it has recently been reported that foreign Ag-reactive T cells primed using CFA differentiate into Th1 effectors, whereas T cells of the same specificities primed using IFA default to a Th2 lineage (13), we tested whether T cells from donors immunized with MBP_{87-106} in IFA produce IL-4 and IL-10 on in vitro challenge. We were unable

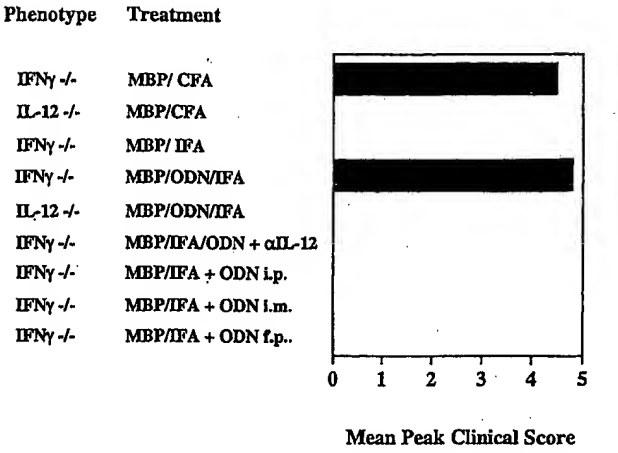

FIGURE 2. C57BL/6 IFN-$\gamma^{-/-}$ but not IL-$12^{-/-}$ mice are susceptible to EAE by active immunization with MBP and CpG ODN. Autoantigens and microbial adjuvants must be delivered to the same site for effective disease induction. C57BL/6 IFN-$\gamma^{-/-}$ or IL-$12^{-/-}$ mice were immunized s.c. with two doses of bovine MBP (100 μg/injection) emulsified with an equal volume of CFA, IFA plus CpG ODN (60 μg/dose), or IFA alone, separated by 1 wk. Some animals immunized with MBP-IFA were simultaneously injected with CpG ODN (60 μg in 0.2 ml PBS) by the i.p., i.m., or footpad (f.p.) route. Some of the mice immunized with MBP-ODN-IFA were injected with a neutralizing mAb, C17.8, to IL-12 (0.5 mg/injection) on days 0, 3, 6, and 9 after the first immunization. Animals injected with control rat IgG according to the same schedule resembled mice that did not receive any Ab treatment (not shown). All groups were monitored on a daily basis from days 0 to 30 and rated on a 5-point scale as described in the text. The clinical scores shown were measured between days 13 and 16 at the peak of disease. The results were pooled from three experiments with four to eight mice per group.

to detect either cytokine in the supernatants of any of the cultures in multiple experiments (data not shown).

An alternative possibility was that MBP-reactive T cells primed with IFA alone were in an intermediate stage of development that would allow them to mature into Th1 encephalitogenic effector cells after reactivation under Th1 polarizing conditions. Expression of the β2 subunit of the IL-12 receptor (IL-12Rβ2) is a critical step in Th1 differentiation (30). Furthermore, we have found that the ability of myelin protein-specific $CD4^+$ T cells to induce EAE correlates with their ability to up-regulate IL-12Rβ2 on antigenic stimulation (29). We therefore compared IL-12Rβ2 expression in LN cells from animals immunized with MBP_{87-106}/IFA or MBP_{87-106}/CpG ODN/IFA after restimulation with Ag in vitro. T cells from animals primed with Ag in CpG ODN/IFA significantly up-regulated IL-12β2 mRNA upon in vitro challenge with Ag alone. IL-12Rβ2 mRNA expression was dependent on the presence of endogenous IL-12 because it was abrogated by the addition of anti-IL-12. By contrast, IL-12Rβ2 mRNA was only modestly induced by the reactivation of T cells that had been primed with MBP_{87-106} in IFA only. However, when recombinant IL-12 was added, these T cells up-regulated IL-12Rβ2 mRNA expression and secreted IFN-γ to levels comparable with those of MBP-reactive T cells that had been exposed to CpG ODN in vivo (Fig. 3, *A* and *B*). More importantly, the MBP_{87-106}/IFA primed LN cells were able to transfer moderate-severe EAE in 100% of naive syngeneic recipients after stimulation with IL-12 (Fig. 3*C*).

Induction of IL-12Rβ2 expression is critically dependent on the presence of IL-12 as T cells from IL-$12^{-/-}$ mice do not express the IL-12Rβ2 after stimulation with specific Ag or polyclonal stimulation with anti-CD3 (Ref. 29 and our unpublished data). The

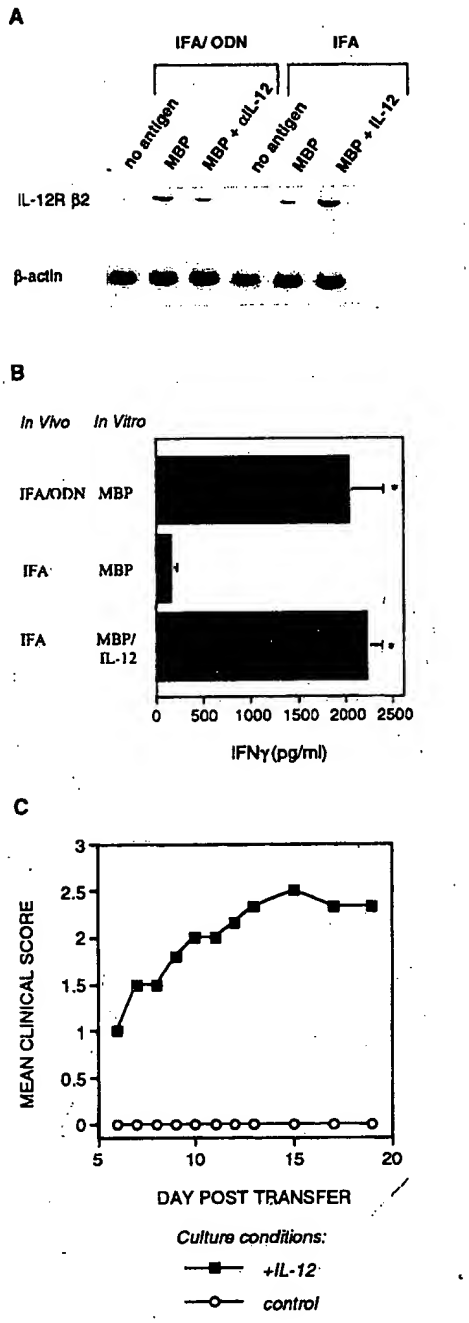

FIGURE 3. MBP-reactive LN cells from IFA-immunized mice retain the capacity to up-regulate IL-12Rβ2 chain and to differentiate into Th1 pathogenic effector cells after reactivation in the presence of IL-12. Draining LNs were removed from mice immunized with MBP_{87-106} (in either IFA or IFA plus CpG ODN) as described in Fig. 2. A, LN cells were cultured with or without Ag. Recombinant murine IL-12 (20 ng/ml) or a neutralizing mAb against IL-12 (αIL-12) (C17.8, 10 μg/ml) were added to some wells as indicated in the figure. At 72 h, cells were harvested, and RNA was isolated. Northern blot analysis was performed using probes specific for murine IL-12Rβ2 chain and, subsequently, for β-actin. Data are representative of three experiments with similar results. *B*, Supernatants from some of the cultures described in *A* were collected at 72 h for IFN-γ quantification by sandwich ELISA. The results represent the difference between cytokine production in the presence or absence of Ag, respectively. Means ± SD of three experiments are shown (*, $p < 0.005$). *C*, LN cells from donors immunized with MBP_{87-105} in IFA were challenged with Ag in the presence ([squlf) or absence (○) of recombinant IL-12 (20 ng/ml), harvested at 96 h, washed extensively, and injected i.p. into naive syngeneic recipients (50×10^6 cells/mouse). Recipients were followed on a daily basis and rated for neurological impairment on a 5-point scale as described in the text. The results shown are pooled from 2 experiments with a total of 10–12 mice/group.

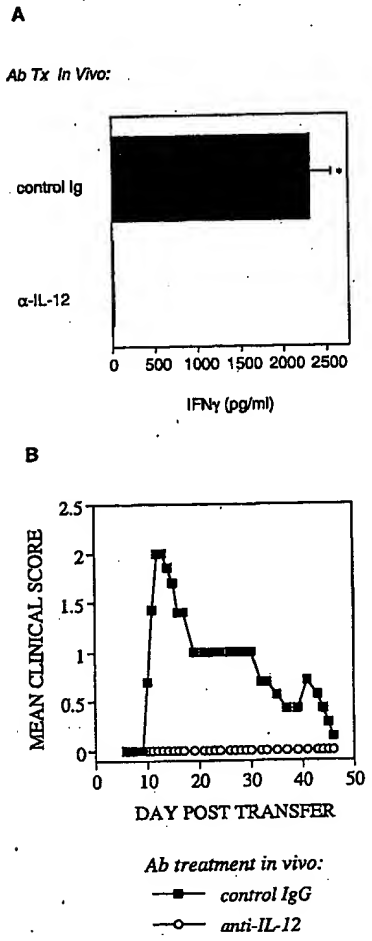

FIGURE 4. Exposure to endogenous IL-12 during priming with MBP_{87-106} in IFA is required for differentiation to the pre-Th1 phenotype. SJL mice (20–30/group) were immunized with MBP_{87-106} in IFA s.c. and injected i.p. with either rat IgG or a neutralizing mAb to IL-12 on days 0 (1 mg/mouse), 3 (0.5 mg), and 6 (0.5 mg). LN cells were harvested on day 10 and cultured with MBP_{87-106} and IL-12 (20 ng/ml). *A*, Supernatants were collected at 72 h for IFN-γ quantification by sandwich ELISA. The results represent the difference between cytokine production in the presence or absence of Ag, respectively. Means ± SD of three experiments are shown (*, $p < 0.005$). *B*, LN cells were collected at 96 h, washed, and injected i.p. into naive syngeneic recipients (60×10^6 cells/mouse). Recipients were monitored on a daily basis and rated for clinical signs as described in Fig. 3*C*. The results shown are pooled from two experiments with five to seven mice per group.

presence of low, but detectable, levels of IL-12Rβ2 on T cells from mice primed with MBP/IFA (Fig. 3, *lane 5*) raised the possibility that immunization with Ag in IFA stimulated a low level of IL-12 production, sufficient to induce modest IL-12Rβ2 expression by the responder T cells but not sufficient to fully drive their differentiation along a Th1 lineage. To test this hypothesis, we immunized SJL mice with MBP_{87-106} in IFA and simultaneously injected either a neutralizing Ab against IL-12 or an isotype-matched control Ab. Ten days later, draining LN were removed and stimulated with MBP_{87-106} and recombinant IL-12. Whereas MBP-reactive LN cells from control Ab-treated donors secreted IFN-γ and transferred EAE to naive recipients after reactivation with IL-12, LN cells from anti-IL-12-treated donors, that were restimulated in the presence of IL-12, failed to produce IFN-γ and were unable to induce EAE on passive transfer (Fig. 4). LN cells from anti-IL-12-treated donors had not been deviated to a Th2

phenotype because they failed to produce IL-4 or IL-10 (data not shown). We conclude from these studies that small quantities of IL-12, produced either constitutively or in response to immunization, play a critical role in shaping the phenotype of MBP-reactive T cells primed with Ag in IFA alone; immunization by this route does not appear to result in the differentiation of autoreactive cells into either Th2 or Tr1 (31) cells but appears to partially drive them along the Th1 pathway as demonstrated by their ability to up-regulate IL-12Rβ2 and differentiate into Th1 autoimmune effectors when reactivated in vitro in the presence of IL-12.

Discussion

The nature of individual Ag-specific immune responses is determined by a collaboration between the innate and adaptive immune systems. A variety of nonspecific signals delivered by live microbes and tumor cells in physiological settings and by adjuvants in experimental settings induce functional changes in dendritic cells and macrophages, the so-called "professional" APCs. This, in turn, influences the path subsequently taken by T cells activated in the same microenvironment. For example, a number of microbial preparations, such as heat-killed *M. tuberculosis*, stimulate IL-12 production by APCs. T cells activated by those APCs are thereby biased toward a Th1 lineage. Normally, this type of cross-talk is beneficial to the host; Th1 responses are most effective in eradicating mycobacterial infections (32). However, when the innate response becomes subverted to guide the development of autoreactive T cells, organ-specific autoimmune diseases may result. In this paper, we demonstrate that IFA containing a 20-base-long ODN with two unmethylated CpG dinucleotides is as effective an adjuvant as CFA in promoting the development of encephalitogenic T cells in vivo. These findings confirm and extend earlier reports of induction of potent Th1 responses to foreign Ags using a similar protocol with CpG ODN in IFA (28, 33, 34).

There is a wealth of circumstantial evidence supporting the conclusion that the major, if not the only, role of microbial adjuvants in EAE is to induce IL-12 production by APC during a crucial stage in the development of myelin-reactive T cells. We, as well as others, have established the importance of IL-12 in the pathogenesis of EAE (22–26). Furthermore, the disease-promoting actions of both CpG ODN/IFA and CFA correlate with their capacity to induce autoreactive Th1 differentiation, and neither adjuvant is effective in the absence of IL-12. Nonetheless, it is still possible that CpG ODNs stimulate APCs to produce other soluble factors such as type I IFNs or IL-18 (34–37) and/or induce the expression of cell surface molecules such as MHC class II or CD80/CD86 (38) that act synergistically with IL-12 during autoimmune pathogenesis. It has also been recently reported that CpG ODNs provide potent growth and maturation signals for dendritic cells (39, 40), have direct effects on NK cells and B cells (41–43), and may have APC-independent effects on T cells (41, 44). The experimental model described in this study should prove to be very useful in determining which of the pleiotropic effects of CpG are most important in the activation of autoreactive T cells

For a control group in all of our studies, we immunized animals with Ag in IFA alone. IFA contains only mineral oil, which functions as a local Ag depot. T cells isolated from the draining LN of IFA immunized animals mounted as strong an Ag-specific lymphoproliferative response as T cells isolated from CFA-primed mice. Similar results were recently reported by other groups (13, 33). By contrast, several earlier studies found that immunization with Ags in the absence of microbial adjuvants lead to deletion, anergy, or active suppression of the targeted T cell population (14, 16, 45–49). In most of the latter studies, Ags were administered in relatively large quantities and delivered by alternate routes when used as a tolerogen in IFA as opposed to as an immunogen in CFA. The paradoxical outcomes may have resulted from the targeting of different classes of APCs in the spleen and LN, respectively.

Chu et al. (28) demonstrated that immunization of mice with Ag in IFA, as opposed to CFA or CpG ODN/IFA, resulted in the production of comparable amounts of total Ag-specific IgG but failed to generate IFN-γ producing LN cells (28). These results and others (13, 15) have fostered the concept that T cells primed by Ag in CFA exclusively differentiate along a Th1 pathway, whereas those primed by Ag in IFA default to a Th2 lineage. In many of these studies, a Th2 response is defined by the expansion of IL-5-producing effector cells in the setting of a stable or diminished IFN-γ-producing population, and the induction of specific IgG1 but not IgG2a Abs. Our results strongly suggest that the concept that immunization with Ag in IFA results in a polar Th2 response is an oversimplification. T cells harvested from the draining LNs of MBP_{87-106}/IFA-primed mice appear to be in a transitional state and have yet to undergo terminal differentiation along a conventional Th pathway. After challenge in vitro, these cells do not produce detectable levels of IFN-γ, IL-4, or IL-10. Unlike Th2 cells, they express IL-12Rβ2, but at a level that is considerably lower than that expressed by typical Th1 cells primed with CpG ODN or CFA. Nevertheless, the level is sufficient to permit Th1 autoimmune effector differentiation after exposure to high doses of exogenous IL-12 in vitro. The capacity of anti-IL-12 to prevent the development of this unique population of cells after immunization with Ag in IFA contradicts the view that they are Th2 precursors because IL-12 antagonizes, rather than promotes, Th2 differentiation (50).

One possible explanation for the differences between our results and those of Yip et al. (13) is that we have studied the response to an autoantigen, whereas they studied responses to foreign Ags. Autoreactive T cells that escape negative selection in the thymus tend to bear TCRs with a relatively low affinity (51). It is possible that the foreign Ag-reactive T cells which bear higher affinity TCR are more likely to produce IL-4 during in vivo priming in the absence of microbial adjuvants and, consequently, to commit to a Th2 phenotype. If lower affinity autoreactive T cells fail to secrete IL-4, they may maintain IL-12 receptor expression (although at a relatively low level) and subsequently be able to differentiate into Th1 cells when reactivated under polarizing conditions. Alternatively, immunization with Ag in IFA may result in a mixed Th response with partial activation of Th1 cells and more complete activation of Th2 cells irrespective of the nature of the Ag used as an immunogen.

Myelin-reactive T cells exist in healthy individuals (52) and remain a potential reservoir of pathogenic effectors which, when appropriately stimulated, could precipitate an autoimmune state. We have previously characterized a population of T cells in B.10.S mice after immunization with MBP in CFA (23, 29). These cells closely resemble the population of T cells generated by immunization of SJL mice with MBP in IFA. Both populations fail to produce IFN-γ in vitro when restimulated with Ag, fail to up-regulate their IL-12Rβ2 chain, and fail to transfer EAE. Moreover, after exposure to IL-12, both differentiate into pathogenic Th1 effector cells. Because some myelin Ags are expressed in peripheral sites (53), it is possible that in healthy individuals similar "benign" populations of autoreactive effector cells are activated in a noninflammatory setting at some point during their life spans. If this occurs, they would acquire the characteristics of memory cells and thereby resemble the "pre-Th1" cells we have described. Our data suggest that such T cells may be capable of fully differentiating into Th1 effectors on reactivation in an inflammatory milieu. Hence, our findings may explain, in part, the association between autoimmune episodes and infectious illnesses/vaccinations (1–9). They also raise a cautionary note regarding the use of DNA vaccinations with vectors containing immunostimulatory CpG motifs due

to the possibility of triggering autoimmune phenomena in a predisposed individual. It has recently been shown that bacterial DNA can exacerbate Theiler's virus murine encephalitis and can enhance relapsing remitting EAE induced by immunization with proteolipid protein (54).

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Effect of Suppressive DNA on CpG-Induced Immune Activation[1]

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Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs stimulate a strong innate immune response. This stimulation can be abrogated by either removing the CpG DNA or adding inhibitory/suppressive motifs. Suppression is dominant over stimulation and is specific for CpG-induced immune responses (having no effect on LPS- or Con A-induced activation). Individual cells noncompetitively internalize both stimulatory and suppressive ODN. Studies using ODN composed of both stimulatory and suppressive motifs indicate that sequence recognition proceeds in a 5′→3′ direction, and that a 5′ motif can block recognition of immediately 3′ sequences. These findings contribute to our understanding of the immunomodulatory activity of DNA-based products and the rules that govern immune recognition of stimulatory and suppressive motifs. *The Journal of Immunology,* **2002, 169: 5590–5594.**

Bacterial DNA contains bioactive CpG motifs that interact with Toll-like receptor 9 to trigger an innate immune response (1–6). While CpG-induced immunity helps protect the host from pathogenic infections (7–10), exposure to stimulatory motifs can have deleterious consequences, ranging from autoimmune disease to death (11–15).

Krieg et al. (16) were the first to report that neutralizing or suppressive motifs can selectively block CpG-mediated immune stimulation. These motifs inhibited cytokine production in vitro and reduced the adjuvant effects of CpG DNA in vivo. Suppressive motifs are rich in polyG or -GC sequences, tend to be methylated, and are present in the DNA of mammals and certain viruses (16–18).

Little is known about the kinetics, magnitude, or nature of the immune inhibition elicited by suppressive motifs. Current studies establish that the immunostimulatory activity of CpG DNA can be reversed within several hours by removal of stimulatory DNA or addition of suppressive DNA. Stimulatory and suppressive DNA binds to and interacts with the same cells. When both sequence types are present on a single strand of DNA, recognition proceeds in a 5′→3′ direction. Suppression is generally dominant over stimulation, although a motif in the 5′ position can interfere with recognition of a motif immediately downstream. Understanding the rules governing cellular responses to stimulatory and suppressive motifs should facilitate the design of oligodeoxynucleotides (ODN)[3] for therapeutic uses.

Materials and Methods

Animals

Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions and were used at 8–20 wk of age. All studies involved protocols approved by the Center for Biologics Evaluation and Research animal care and use committee.

Oligodeoxynucleotides

Studies used phosphorothioate-modified ODNs that were synthesized at the Center for Biologics Evaluation and Research core facility (19). The following ODNs were used: immunostimulatory, ODN_{1466} (TCAACGTTGA) and ODN_{1555} (GCTAGACGTTAGCGT); control, ODN_{1471} (TCAAGCTTGA) and ODN_{1612} (GCTAGAGCTTAGGCT); and suppressive, ODN_{1502} (GAGCAAGCTGGACCTTCCAT) and ODN_{H154} (CCTCAAGCTTGAGGGG). The underlined bases represent the 10-mer sequences that were incorporated into complex multideterminant ODN used in some experiments. There was no detectable protein or endotoxin contamination of these ODN.

Mammalian DNA was purified from BALB/c spleens (Wizard Genomic DNA purification kit; Promega, Madison, WI). *Escherichia coli* DNA was obtained from Life Technologies (Gaithersburg, MD). Endotoxin contamination in these preparations was <0.1 U/ml after purification (20). Double-stranded DNA was converted to ssDNA by heat denaturing at 95°C for 5 min, followed by immediate cooling on ice.

Cytokine ELISAs

Spleen single-cell suspensions were washed three times and resuspended in RPMI 1640 supplemented with 5% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml of penicillin/streptomycin. Cells (5×10^5/well) were cultured in flat-bottom microtiter plates (Costar, Corning, NY) with 1 μM ODN for 18–24 h. Culture supernatants were collected, and cytokine levels were measured by ELISA. In brief, 96-well Immulon H2B plates (Thermo LabSystems, Franklin, MA) were coated with cytokine-specific Abs and blocked with PBS 1% BSA as previously described (21). Culture supernatants were added, and bound cytokine was detected by the addition of biotin-labeled secondary Abs, followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (PNPP; Pierce, Rockford, IL). Standard curves were generated using recombinant cytokines. The detection limit for these assays was 0.8 U/ml for IFN-γ, 0.1 ng/ml for IL-6, and 0.1 ng/ml for IL-12. All assays were performed in triplicate.

Cytokine-specific ELISPOT assays

A spleen single-cell suspension prepared in RPMI 1640 plus 5% FCS was serially diluted onto plates precoated with anti-cytokine Abs (21). Cells were incubated with 1 μM ODN at 37°C for 8–12 h, and the secretion of cytokine was detected colorimetrically as previously described (21).

Cell surface binding and internalization of ODN

Spleen cells (2×10^6/ml) were incubated with 1 μM of unlabeled and/or fluorescent-labeled ODN for 10 min at 4°C (binding experiments) or for 1 h at 37°C (uptake experiments). Cells were washed, fixed, and analyzed by FACScan (BD Biosciences, San Jose, CA) (22).

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide.

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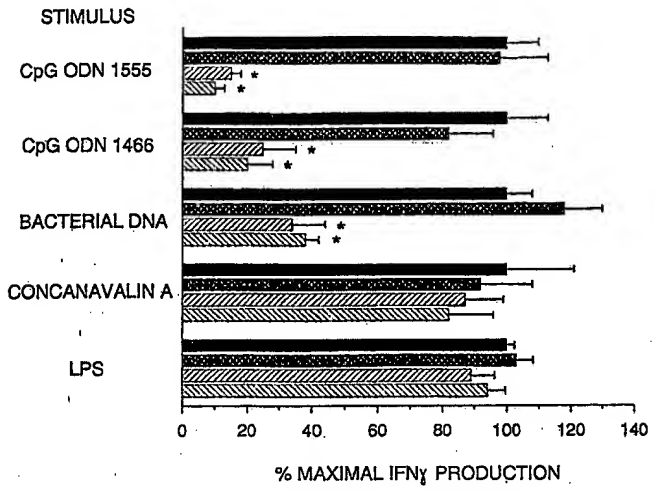

FIGURE 1. Effect of suppressive ODN on CpG DNA and mitogen-induced IFN-γ production. BALB/c spleen cells were stimulated with 1 μM CpG ODN (ODN_{1555}, ODN_{1466}), 50 μg/ml of bacterial DNA, 5 μg/ml of Con A, or 5 μg/ml of LPS. The response of these cultures (■) was compared with that of cells costimulated with 1 μM control ODN_{1612} (▩), suppressive ODN_{1502} (▨), or suppressive ODN_{H154} (▧). The number of IFN-γ-secreting cells was determined by ELISPOT after 18 h. Data represent the average ± SD of triplicate cultures. The experiment was repeated three times with similar results.

Statistical analysis

Statistically significant differences between two groups were determined using the Wilcoxon rank-sum test. When comparing more than two groups, differences were determined using a two-tailed nonparametric ANOVA with Dunn's post-test analysis. A value of $p < 0.05$ was considered significant.

Results

Mammalian DNA suppresses CpG DNA-induced immune activation

Single-stranded bacterial DNA and synthetic ODN containing unmethylated CpG motifs stimulate immune cells to mature, proliferate, and produce cytokines, chemokines, and Ig (2–5). These effects can be blocked by polyG- and/or GC-rich DNA motifs (16, 23). Scores of ODNs were synthesized and tested to identify motifs that selectively inhibited CpG-induced immune responses. The

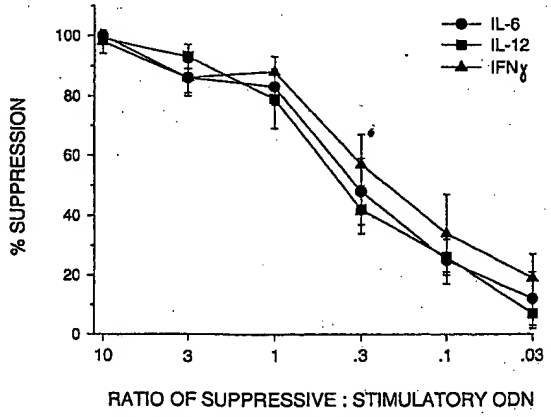

FIGURE 2. Concentration effects of suppressive ODN. BALB/c spleen cells were stimulated with 1 μM CpG ODN_{1555} or ODN_{1466} plus increasing amounts of suppressive ODN_{1502} or ODN_{H154}. Cytokine levels in culture supernatants were measured by ELISA after 24 h. Results represent the mean ± SD of four different experiments.

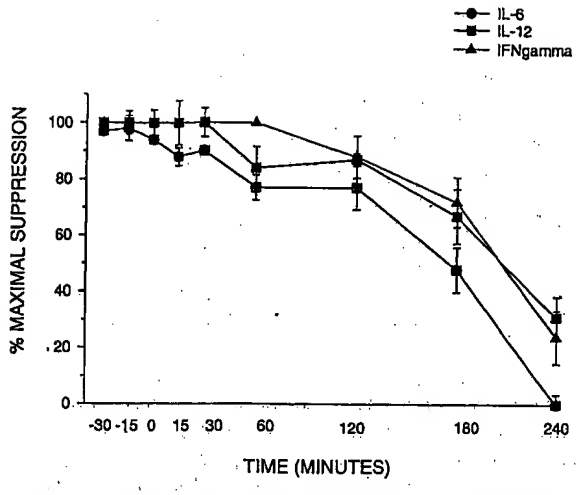

FIGURE 3. Kinetics of suppressive ODN. BALB/c spleen cells were stimulated with 1 μM CpG ODN_{1555}. At various times, 1 μM suppressive ODN_{1502} was added. Cytokine levels in supernatants were measured by ELISA after 24 h. Results represent the mean of two independent experiments.

two most active of these suppressive ODN (ODN_{1502} (GAG CAAGCTGGACCTTCCAT) and ODN_{H154} (CCTCAAGCTT GAGGGG)) were selected for detailed study. As shown in Fig. 1, these suppressive ODN blocked a majority of the IFN-γ production induced by bacterial DNA or CpG ODN ($p < 0.01$). Suppressive ODN were neither toxic nor broadly immunosuppressive, as they did not interfere with the mitogenic activity of LPS or Con A (Fig. 1 and data not shown).

The activity of suppressive ODNs was concentration dependent, with 50% suppression being achieved at a suppressive ODN:CpG ODN ratio of ~1:3 (Fig. 2). To examine the kinetics of this inhibition, suppressive ODN were added to BALB/c spleen cells at various times after CpG-induced stimulation. Maximal inhibition was observed when suppressive ODN were coadministered with CpG ODN, although statistically significant inhibition persisted when suppressive ODN were added up to 3 h later (Fig. 3). These findings suggest that CpG-induced immune activation is an ongoing process and can be inhibited after the stimulatory signal is delivered.

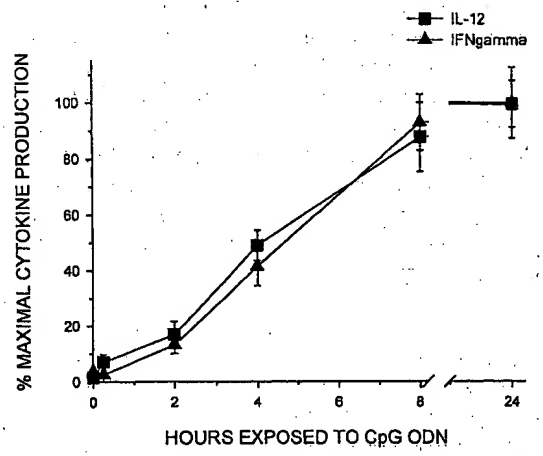

FIGURE 4. Effect of removing CpG ODN from cultured cells. CpG ODN_{1555} (1 μM) was added to BALB/c spleen cells at time zero. The cells were washed free of this ODN after various incubation periods. IFN-γ and IL-12 levels in culture supernatants were measured by ELISA after 24 h. Results represent the average ± SD of duplicate cultures. Similar results were obtained in studies of CpG ODN_{1466}.

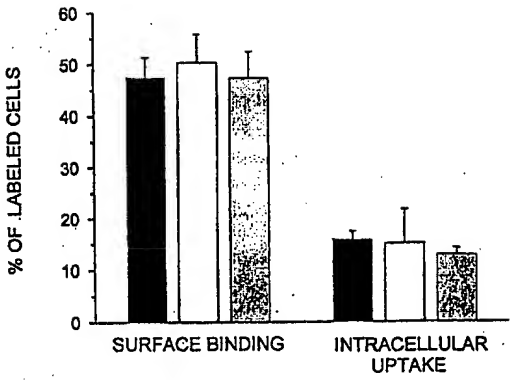

FIGURE 5. Suppressive ODN do not block the binding or uptake of CpG ODN. BALB/c spleen cells were incubated with 1 μM CpG ODN_{1555} (■) plus 1 μM suppressive ODN_{1502} (▨) or control ODN_{1612} (□) for 2 h. The percentage of cells that bound or internalized the CpG ODN was determined by FACS. Similar results were obtained using CpG ODN_{1466}, suppressive ODN_{H154}, and control ODN_{1471}.

To test this conclusion, spleen cells were incubated with CpG ODN for various periods, and cytokine production was analyzed after 24 h. Cells stimulated with CpG DNA for 8 h produced 90% as much cytokine as cells stimulated continuously for 24 h (Fig. 4). Cells treated with CpG ODN for only 4 h produced half as much cytokine, while cells treated with CpG DNA for ≤2 h showed only minimal activation (Fig. 4). These findings support the conclusion that CpG-induced cellular activation is reversible for several hours.

Suppressive ODN do not block CpG ODN uptake or induce the production of inhibitory factors

The results described above indicate that CpG-induced immune activation can be reversed either by adding suppressive ODN or by removing stimulatory ODN. This suggests that suppressive ODN might block the ongoing uptake of CpG DNA. Yet FACS analysis demonstrated that neither cell surface binding nor internalization of FITC-labeled CpG ODN was significantly reduced by suppressive ODN at concentrations that blocked cytokine production by ~75% (Fig. 5 and data not shown). Moreover, precisely the same cells that bound and internalized CpG ODN interacted with suppressive ODN (Fig. 6).

The possibility that suppressive motifs might induce the production of a factor that blocked CpG-dependent immune stimulation was then investigated. Initial studies established that BALB/c spleen cells preincubated with suppressive ODN remained unresponsive to CpG-induced stimulation for several hours (Table I, line 3). If this nonresponsive state was mediated by a soluble factor (or inhibitory cell-cell interactions) then cells pretreated with suppressive ODN should block CpG-induced stimulation of naive splenocytes. As shown in Table I, cells treated with suppressive ODN had no significant effect on CpG-dependent cytokine production by fresh spleen cells.

Table I. *Effect of mixing cells treated with suppressive vs stimulatory ODN*[a]

Suppressive ODN		ODN Added During Culture	% Maximal Cytokine Production	
Pretreatment	Fresh Cells		IL-6	IL-12
−	+	CpG	100 ± 13	100 ± 6
−	+	Control	3 ± 2	7 ± 2
+	−	CpG	9 ± 6	6 ± 2
+	−	Control	0 ± 0	0 ± 0
+	+	CpG	86 ± 16	105 ± 12
+	+	Control	0 ± 0	0 ± 0

[a] BALB/c spleen cells were treated with 1 μM suppressive ODN_{H154} for 2 h and then washed (first column). These cells were added to naive splenocytes (second column) plus 1 μM control (ODN_{1471}) or CpG (ODN_{1555}) ODN. IL-6 and IL-12 levels in culture supernatants were measured by ELISA after 18 h. The percentage of maximal cytokine production was calculated by the formula: (cytokine produced by treatment group) − (background)/(cytokine produced by fresh cells stimulated with CpG ODN) − (background) × 100%, where the background was cytokine levels in fresh cells cultured in medium alone. Results represent the average ± SD of triplicate assays, each standardized to the response induced by bacterial DNA (62 pg/ml IL-6; 134 pg/ml IL-12).

Cellular recognition of suppressive vs stimulatory motifs

The above studies establish that suppressive motifs on one strand of DNA block the immune activation induced by stimulatory motifs on a different strand (i.e., *trans*-suppression). To better understand the interaction between suppressive and stimulatory motifs, ODNs containing both were synthesized. A set of four 20-mer ODNs was constructed in which one of two different CpG motifs was placed immediately 5′ to either of two suppressive motifs (referred to as [CpG-Sup] ODN).

All four of these [CpG-Sup] ODN were stimulatory, triggering murine spleen cells to produce IL-6, IL-12, and IFN-γ to the same extent as an ODN of the same length in which the suppressive motif was replaced by a control sequence (i.e., one that was neither stimulatory nor suppressive; Table II). [CpG-Sup] ODNs did not inhibit the immune activation induced by an independent CpG ODN (Table II). These results suggest that a suppressive motif is inactive when located immediately 3′ to a CpG motif on the same strand of DNA.

To better understand this phenomenon, longer ODNs were synthesized in which the CpG and suppressive motifs were separated by progressively longer CT spacers. Adding a 5-base spacer generated an ODN that was still stimulatory (Table III). However, separating the motifs by ≥10 bases yielded ODNs that were suppressive, demonstrated by their ability to block the stimulatory activity of coadministered CpG ODNs (Table III). The trivial possibility that the CT spacer somehow reduced CpG activity was eliminated by substituting a control motif for the 3′ suppressive motif. The resulting ODNs were fully stimulatory (Table III and data not shown).

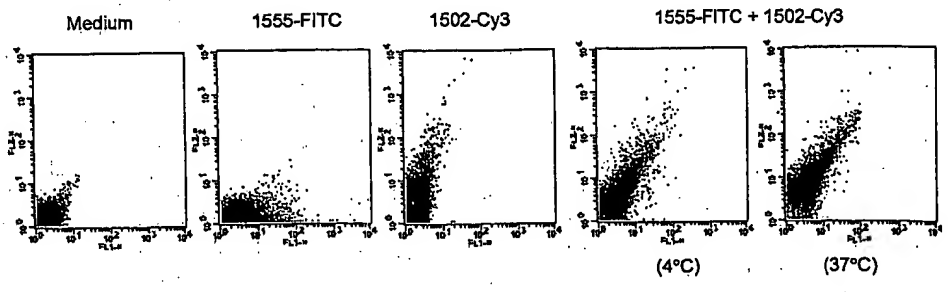

FIGURE 6. Binding and internalization of suppressive and CpG ODN. BALB/c spleen cells were incubated with 1 μM CpG ODN_{1555} and/or 1 μM suppressive ODN_{1502} at 4°C for 10 min or at 37°C for 2 h. Note that the same cells bound and internalized both CpG and suppressive ODN. Binding increased as the time of incubation was prolonged (Fig. 5).

Table II. *Effect of motif position on immunostimulatory activity*[a]

Location of Motifs (5′→3′)	No. of Cytokine-Secreting Cells		
	IL-6	IL-12	IFN-γ
CpG ODN[b]	79 ± 3	1980 ± 230	260 ± 40
[CpG-Sup] ODN[b]	72 ± 14	2080 ± 480	230 ± 60
[Sup-CpG] ODN	0 ± 0	140 ± 30	0 ± 0
[CpG-Cont] ODN[b]	64 ± 12	2210 ± 130	284 ± 34
[Cont-CpG] ODN[b]	80 ± 11	1942 ± 88	238 ± 28
[Cont-Sup] ODN	8 ± 2	184 ± 34	36 ± 8
[CpG-Sup] ODN + Sup ODN	4 ± 2	226 ± 38	28 ± 6
[Sup-CpG] ODN + CpG ODN	7 ± 3	250 ± 32	34 ± 9

[a] BALB/c spleen cells (10^6) were coincubated with 1 μM of each ODN. Complex ODN (20 bp in length) were constructed from 10-mer encoding suppressive (Sup; GAGCAAGCTG and AGCTTGAGGG), stimulatory (CpG; TCACGTTGA and TA GACGTTAG), or control (Cont; TCAAGCTTGA and TAGAGCTTAG) motifs. The number of cytokine-secreting cells per 10^6 cells was determined by ELISPOT after 24 h of stimulation. Results represent the average ± SD of triplicate assays involving at least two ODN of each type.

[b] Stimulatory ODN, $p < 0.05$.

The impact of placing a suppressive motif 5′ to a CpG motif was then examined. ODNs with a suppressive motif in the 5′ position induced little or no immune activation even when the CpG motif was shifted up to 20 bp downstream from the suppressive motif (Tables II and III). This lack of activity could not be attributed to the 3′ location of the CpG motif, since CpG ODNs with a control sequence at the 5′ end were stimulatory. All ODNs containing a suppressive motif in the 5′ position also inhibited the stimulatory activity of a coadministered CpG ODN (Tables II and III). These findings suggest that the relative positions of stimulatory and suppressive motifs determine the immunomodulatory properties of DNA.

Discussion

DNA has multiple and complex effects on the immune system. The innate immune response triggered by unmethylated CpG motifs present in bacterial DNA improve host resistance to infectious pathogens (7, 9, 10, 24). Yet CpG stimulation can increase the host's susceptibility to autoimmune disease and death (11–14, 25, 26). This work examines the ability of suppressive motifs to specifically down-regulate CpG-induced immunity.

Previous studies established that CpG DNA interacts with TLR9 to trigger the translocation of NF-κB from the cytoplasm to the nucleus and the subsequent up-regulation of cytokine gene expression (1, 6, 27–30). Current results demonstrate that this is not an all-or-none phenomenon. Although NF-κB translocation is initiated within minutes of CpG administration (29), the subsequent increase in cytokine production occurs over a period of hours (2) and is significantly reduced by the addition of suppressive ODN or the removal of stimulatory CpG DNA (Figs. 3 and 4). Consistent with these findings, suppressive motifs were recently shown to down-regulate CpG-dependent NF-κB and AP-1 induction (17, 18). These observations suggest that CpG motifs must continuously signal receptive cells for triggering to persist.

The sequence and length of a DNA strand determine its activity. By synthesizing and testing scores of ODNs, our laboratory and that of Krieg et al. independently identified G- and GC-rich motifs that selectively block CpG-dependent activation (16). Of note, Zhao et al. (31) showed that not all GC-rich repeats confer suppressive activity, while Halpern et al. (32) showed that ODNs containing runs of >15 polyGs can inhibit both CpG- and mitogen-induced immune responses. Suppressive activity appears to depend upon an ODN's secondary/tertiary structure, although sequence-nonspecific competition for ODN uptake is also possible (28). In this context, G-rich regions facilitate the formation of complex intra- and interchain Hoogsteen hydrogen bonds (33, 34). Depending on how these chains fold, activity may be gained or lost.

To validate the findings in this report, all experiments were repeated with multiple ODNs containing different combinations of suppressive and/or CpG motifs. In addition, the critical role of the suppressive motifs was established by showing that control motifs neither enhanced nor prevented CpG induced immune stimulation.

Table III. *Effect of distance between motifs on ODN activity*[a]

ODN	Cytokine-Producing Cells (% maximum)		
	IL-6	IL-12	IFN-γ
CpG ODN[b]	100 ± 11	100 ± 7	100 ± 10
CpG ODN[b] + Cont ODN	97 ± 14	98 ± 9	100 ± 17
CpG ODN[b] + Sup ODN	16 ± 6	21 ± 6	18 ± 5
LPS	100 ± 2	99 ± 2	100 ± 2
LPS + Sup ODN	94 ± 7	94 ± 5	92 ± 7
[CpG-Sup] ODN[b]	87 ± 12	>100 ± 14	92 ± 14
[CpG-5 bases-Sup] ODN[b]	>100 ± 4	>100 ± 21	>100 ± 22
[CpG-10 bases-Sup] ODN	38 ± 6	64 ± 15	42 ± 7
[CpG-20 bases-Sup] ODN	7 ± 4	48 ± 13	24 ± 8
[CpG-Cont] ODN[b]	94 ± 7	>100 ± 14	99 ± 11
[Sup-CpG] ODN	0 ± 0	0 ± 0	0 ± 0
[Sup-20 bases-CpG] ODN	8 ± 5	9 ± 3	2 ± 1
[CpG-Sup] ODN[b] + CpG ODN[b]	>100 ± 16	>100 ± 15	98 ± 13
[CpG-5 bases-Sup] ODN[b] + CpG ODN[b]	>100 ± 18	>100 ± 11	98 ± 20
[CpG-10 bases-Sup] ODN + CpG ODN[b]	58 ± 7	75 ± 9	66 ± 9
[CpG-20 bases-Sup] ODN + CpG ODN[b]	27 ± 5	26 ± 10	30 ± 8
[Sup-CpG] ODN + CpG ODN[b]	9 ± 4	11 ± 4	8 ± 5
[Sup-20 bases-CpG] ODN + CpG ODN[b]	5 ± 1	9 ± 3	13 ± 2

[a] BALB/c spleen cells were stimulated in vitro with 1 μM of each ODN (or 5 μg/ml LPS), and the number of cells activated to secrete cytokine was determined 8 h later by ELISPOT. The percentage of cells activated to secrete cytokine was calculated by the formula: (number of cells activated by test ODN) − (background)/(number of cells activated by CpG ODN) − (background) × 100%. Multiple combinations of the CpG, suppressive and control 10-mer motifs described in Table II were used in these studies and gave similar results in these experiments. Results represent the average of two to four assays per data point. Table II shows typical numbers of cytokine-secreting cells per 10^6 cells.

[b] Stimulatory ODN, $p < 0.05$.

The data in Tables II and III and Fig. 6 suggest that suppressive and stimulatory motifs are active on the same cells, and that their relative locations on a DNA strand determine the magnitude and nature of the resultant response. The results indicate that 1) cellular recognition of stimulatory and suppressive motifs proceeds in a 5′→3′ direction; and 2) suppression is generally dominant over stimulation, however, 3) when a CpG motif is immediately 5′ to a suppressive motif, stimulation dominates. A likely explanation for the latter phenomenon is that molecules involved in recognizing the 5′ motif block the cell's ability to interact with an immediately adjacent suppressive motif, perhaps due to steric hindrance. When the distance between motifs exceeds 10 bases, this effect dissipates.

Our finding that the relative location of CpG vs suppressive motifs on a single strand of DNA influences the resultant immune response strongly suggests that individual cells recognize both motifs. Experiments using labeled ODNs demonstrate that both types of DNA enter the same cells (Fig. 5 and data not shown). Indeed, the possibility that one type of cell responds only to stimulatory motifs and another only to suppressive motifs is inconsistent with the results in Tables II and III. Moreover, the data shown in Table I indicate that cells exposed to suppressive ODNs do not produce factors or interact on a cell-to-cell basis in such a way as to inhibit other cells from responding to CpG motifs.

Suppressive ODNs could be of use in several therapeutic settings. CpG motifs in antisense and gene therapy vectors contribute to the immune recognition of transfected cells (35). Introducing suppressive sequences 5′ to CpG motifs in these vectors might dampen this immune response and prolong the vector's in vivo activity (16). Alternatively, the immunogenicity of DNA vaccines might be improved by deleting suppressive motifs (16). Finally, suppressive ODN may prove useful in situations where the host's response to bacterial DNA contributes to pathology, as in septic shock or autoimmune disease (11, 25, 36, 37). Since suppressive ODN precisely target the inflammatory response induced by CpG DNA, these therapies may avoid the deleterious side effects associated with generalized immunosuppressive regimens.

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